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Circulating MicroRNAs as Markers of Prostate Cancer Progression: a Time-based Approach

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CIRCULATING MICRORNAS AS MARKERS OF PROSTATE CANCER PROGRESSION: A TIME-BASED APPROACH

Dissertação de Mestrado apresentada ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto em Oncologia — Especialização em Oncologia Molecular

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And now, I am going to tell you a story.

Resumo

Introdução: O cancro da próstata representa uma das doenças oncológicas mais comuns em todo o Mundo, sendo o segundo cancro mais incidente nos homens a nível mundial e o quinto em termos de mortalidade. 80% dos tumores diagnosticados encontram-se ainda confinados ao órgão, pelo que a sua remoção cirúrgica por prostatectomia radical é possível, sendo um tratamento amplamente utilizado. No entanto, em mais de um terço dos pacientes sujeitos a esta terapia surge uma recorrência tumoral que se deverá a pequenas populações de células cancerígenas disseminadas, mas não detectadas, sendo necessários tratamentos adicionais, mais agressivos e que perturbam a qualidade de vida do doente. Na busca por marcadores não-invasivos que possam prever com maior certeza a probabilidade de uma recorrência surgir, os microRNAs surgem como candidatos devido à regulação epigenética que exercem na expressão de genes relacionados com o cancro, estando já descrito o seu papel crítico em doenças do foro oncológico tal como o cancro da próstata.

Objetivos: Assim, o principal objectivo desta Dissertação foi estudar a expressão, no plasma, de dois microRNAs previamente descritos como potenciais oncogenes (miR-375 e miR-182) numa cohort de pacientes submetidos a prostatectomia radical, em dois momentos distintos: antes da cirurgia e durante o tempo de *follow-up* do doente. Posteriormente, procurou-se relacionar a expressão de microRNAs com a expressão dos mesmos microRNAs em tecido tumoral de um sub-grupo de pacientes e também com as variáveis clinicopatológicas de maior relevância.

Material e Métodos: Após a extração de RNA de plasma e de tecido tumoral dos pacientes envolvidos e subsequente quantificação, procedeu-se à síntese de cDNA seguida da medição da expressão relativa do miR-375, do miR-182 e do gene de referência U6 snRNA por qRT-PCR.

Resultados: Confirmou-se que o miR-375 e o miR-182 se encontram sobrexpressos em tecido tumoral comparando com tecido prostático normal. Ambos os microRNAs apresentam também uma maior expressão pré-operatória no plasma em pacientes com tumores em estadios mais elevados. Observou-se ainda que o rácio plasma:tecido da expressão do miR-375 é um preditor independente do tempo de sobrevivência livre de doença. Relativamente aos valores pós-operatórios de microRNAs, verificou-se que a expressão do miR-375 é mais elevada nos pacientes que desenvolveram recorrência bioquímica ou metastização comparando com os que não desenvolveram.

Discussão e Conclusões: O miR-375 desempenha um papel oncogénico em cancro da próstata, encontrando-se sobreexpresso no tecido tumoral comparativamente a tecido normal e também no plasma de doentes com progressão pós-operatória da doença, pelo que a sua expressão na circulação sanguínea pode ser um reflexo da progressão tumoral. Como tal, o miR-375 constitui um possível biomarcador para *follow-up* do paciente após cirurgia. Contrariamente, o papel oncogénico do miR-182 deverá estar limitado aos estágios iniciais da carcinogénese prostática, uma vez que as diferenças de expressão encontradas (tecido e plasma) apenas se manifestam antes da realização da cirurgia e não durante o tempo de *follow-up*.

Summary

Introduction: Prostate cancer is one of the most common malignant diseases in the world, being the second most common cancer in men worldwide and fifth in terms of mortality. 80% of tumors are diagnosed in an organ-confined state, which permits its surgical removal by radical prostatectomy, a widely used treatment. However, a cancer recurrence arises in more than one third of the patients undergoing this therapy, perhaps due to small populations of disseminated cancer cells that remained undetected, therefore requiring additional and more aggressive treatments that disturb patient's quality of life. microRNAs arise as candidates for noninvasive markers that can more accurately predict the likelihood of a recurrence due to their epigenetic regulation they exert in the expression of cancer-related genes as well as their critical role in malignant diseases such as prostate cancer.

Aims: The main goal of this thesis was to study expression of two plasma-circulating microRNAs previously described as potential oncogenes (miR-375 and miR-182) in a cohort of patients undergoing radical prostatectomy at two different moments: at time of surgery and during patient's follow-up time. Subsequently, we investigated for associations between plasma expression of microRNAs and expression of the same microRNAs in tumorous tissue of a subgroup of patients as well as clinicopathological variables of major relevance.

Material and Methods: After RNA extraction from plasma and tumor tissue of patients involved and subsequent RNA quantification, cDNA synthesis was performed followed by measurement of the relative expression of miR-375, miR-182 and the reference U6 snRNA gene by qRT-PCR.

Results: We confirmed that miR-375 and miR-182 are overexpressed in tumor tissue compared to normal prostatic tissue. Both microRNAs also present a higher preoperative plasma expression in patients with higher-grade tumors. It was further observed that the plasma:tissue expression ratio of miR-375 is an independent predictor of disease-free survival time. Regarding the postoperative values of microRNAs, the expression of miR-375 was found to be higher in patients who developed biochemical recurrence or metastasis compared to those who did not.

Discussion and Conclusions: miR-375 plays an oncogenic role in prostate cancer, being overexpressed in the tumor tissue compared to normal tissue and also in plasma of patients with disease progression after therapy, suggesting that its expression in patient bloodstream may be a reflex of tumor progression. As such, miR-375 is a possible biomarker for patient follow-up after surgery. Contrarily, the oncogenic role of miR-182 should be limited to the early stages of prostatic

carcinogenesis, since the expression differences in both tissue and plasma were limited to patients before radical prostatectomy and not during follow-up time.

Table of Contents

Figures Index

Tables Index

List of Abbreviations

AA	African american
ADT	Androgen-deprivation therapy
AFS	Anterior fibromuscular stroma
AJCC	American Joint Committee on Cancer
ANOVA	Analysis of variance
ARRDC3	Arrestin domain containing 3
AS	Active surveillance
AUC	Area under the curve
BCL2	B-cell lymphoma 2
BCR	Biochemical recurrence
BPH	Benign prostatic hyperplasia
BRCA2	Breast cancer 2
CBX7	Chromobox 7
CRPC	Castration-resistant prostate cancer
CYP17	Cytochrome P450 17A1
CYP19	Aromatase cytochrome P450
CZ	Central zone
DNA	Deoxyribonucleic acid
DGCR8	DiGeorge syndrome critical region 8
DRE	Digital rectal examination
EBRT	External-beam radiotherapy
ERG	Erythroblast transformation-specific related gene
FIH1	Factor inhibiting hypoxia-inducible factor 1α
FOXF2	Forkhead box F2
FOXO1	Forkhead box protein O1
GEO	Gene Expression Omnibus
GNA13	G-protein subunit α-13
GG	Grade group
GS	Gleason score

HGPIN	High-grade prostate intraepithelial neoplasia
HIF-1α	Hypoxia-inducible factor 1α
LUTS	Lower urinary tract symptoms
MET	Mesenchymal-epithelial transition
miR	MicroRNA
miRNA	MicroRNA
MNPT	Matched normal prostatic tissue
mRNA	Messenger ribonucleic acid
MRI	Magnetic resonance imaging
MTSS1	Metastasis suppressor protein 1
NDRG1	N-myc downstream regulated gene 1
PBMC	Peripheral blood mononuclear cells
PCa	Prostate cancer
PDH2	Prolyl hydroxylase domain-containing protein-2
PHLPP1	PH domain and leucine-rich repeat protein phosphatase 1
PIN	Prostate intraepithelial neoplasia
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PSA	Prostate-specific antigen
PZ	Peripheral zone
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RECK	Reversion-inducing cysteine-rich protein with kazal motifs
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RP	Radical prostatectomy
Sec23A	Sec23 homolog A (Saccharomyces cerevisiae)
SNAI2	Snail Family Transcriptional Repressor 2
SRD5A2	Steroid α-reductase type 2 gene
snRNA	Small nuclear ribonucleic acid
snoRNA	Small nucleolar ribonucleic acid

suPAR	Soluble urokinase plasminogen activator receptor
TCGA	The Cancer Genome Atlas
TMPRSS2	Transmembrane protease, serine 2
TRBP1	Transactivating response RNA binding protein
TRUS	Transrectal ultrasound
TZ	Transitional zone
UICC	Union for Cancer Control
UTR	Untranslated region
WW	Watchful waiting
XPO5	Exportin 5
YAP-1	Yes associated protein 1
ZEB-1	Zinc finger E-box binding homeobox 1

Introduction

1. The Prostate

1.1. Describing the Prostate Gland

Being an integral part of male reproductive system, the prostate gland is a small exocrine organ located underneath the bladder and in front of the rectum. It is traversed by the urethra and the ejaculatory ducts and plays a crucial role on the nourishment and protection of sperm. During an ejaculation, sperm is released by the testicles and travels through the vas deferens until the seminal vesicles, which secrete a significant proportion of the liquid that becomes the semen. Then the resulting fluid enters the ejaculatory ducts and crosses the prostate gland, which is responsible for the secretion of proteins and hormones which are added to the semen and concomitantly released into the urethra [1].

In an adult male, a healthy prostate gland has an approximate volume of 20 to 25 cm^3 [2] and an estimated weight of 20 grams [3], resembling the size and shape of a walnut. The prostate gland encompasses (1) a stromal component comprised of connective tissue which essentially provides functional support and (2) a glandular component mainly composed of ducts, which allow draining of the prostatic fluid into the urethra, and acini, which are microscopic glands located at the end of the smaller ducts and rounded by secretory epithelial cells responsible for the production of the prostatic contribution to ejaculatory fluid [4]. Both stromal and glandular components are numerously found throughout the prostate gland.

Although a segmentation in lobes was previously thought for human prostate, the homogeneous appearance of prostate surfaces led John E. McNeal to suggest in 1969 a division in four different zones [5], a classification that is widely used nowadays (Figure 1). The peripheral zone (PZ) is comprised of glandular tissue and located at the back of the prostate closest to the rectum. It surrounds the distal urethra, constituting about 70% of the gland and being the origin place of about 75% of carcinomas as well as other prostatic pathologies [6, 7]. The central zone (CZ) is a cone-shaped portion that encircles the ejaculatory ducts and accounts for 25% of the organ. Only about 15% of prostatic cancers arise in CZ, however they tend to be more

aggressive due to their proximity to the seminal vesicles, which increases the likelihood of invasion [8]. Together PZ and CZ represent 95% of the prostatic gland. The transition zone (TZ) is the innermost component of the gland and surrounds the urethra representing only 5% of the organ at puberty. However, TZ tends to continuously enlarge throughout life and thus the development of age-related benign pathologies as well as 10-15% of cancers is frequent in this region. The anterior fibromuscular stroma (AFS) makes up less than 5% of the gland and it is located at the front of the prostate gland closest to the abdomen. It covers the anterior external surface and it is enriched in striated muscle tissue, therefore lacking glandular components [3].

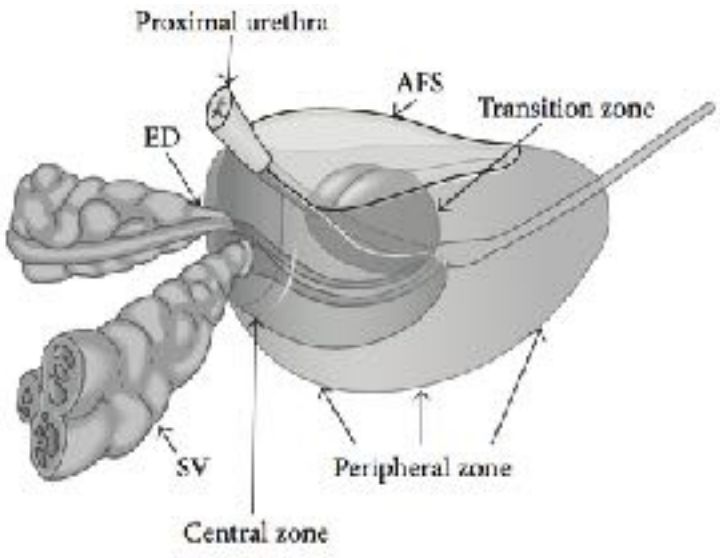


Figure 1: Anatomy of human prostate gland and classification in zones (adapted from [7]). ED = ejaculatory ducts; SV = seminal vesicles; AFS = anterior fibromuscular stroma.

1.2. From Benign Disorders to Prostate Cancer

The normal development and growth of the prostate gland is promoted by the androgen-signaling pathway in which androgens induce gene transcription and subsequent cell proliferation by binding to the androgen receptor (AR) located in cell membrane. Testosterone, mainly produced by testicular Leydig cells is the primary androgen of the human body, however its metabolite dihydrotestosterone (DHT) arises as a more potent androgen with three times higher affinity to the AR and a 5-fold increase in binding time [9]. Testosterone synthesis, conversion to DHT, transport of DHT to target cells and binding to the AR are therefore the main steps of androgen-signaling axis [10]. DHT is thought to maintain the balance between cell proliferation and cell death and gradual disruption of this equilibrium may account for the

progressive enlargement of prostate gland throughout life even during adulthood, thereby explaining the high incidence of prostate growth-related disorders among elderly individuals [10].

One of the most common benign diseases worldwide is benign prostatic hyperplasia (BPH), affecting approximately 50% of men between 51 and 60 years old and 90% of men with more than 80 years old [11, 12]. BPH is characterized by a uncontrolled increase in the number of prostatic cells leading to an overgrowth of nonmalignant tissue and usually arises within the TZ of the prostate gland surrounding urethra thus potentially interfering with the normal flow of urine. In fact, BPH-related lower urinary tract symptoms (LUTS) arise in 25% of men between 50 and 59 years old and 50% of men with more than 80 years old [12] and frequently involve urinating difficulties. As previously reviewed [13], BPH and prostate cancer (PCa) share several traits namely (1) the androgen-dependent growth; (2) the rise with increasing age, with an estimated 15-year lag between both pathologies [12, 14]; (3) common clinical events such as prostatic inflammation (prostatitis), which prompts both BPH [15] and PCa [16] development; and (4) shared genetic alterations that increase the risk of both BPH and PCa such as variants of androgen-related CYP17, CYP19 and SRD5AR genes [17-19]. However, a causal link between BPH and PCa is not clearly characterized. Histologic differences between both pathologies as well as differences in anatomic localization within the prostate gland and the lack of evidence of BPH as an initial disorder in the pathway to PCa suggest that aforementioned associations between BPH and PCa may be due to a coexistence of both pathologies rather than a causal relationship [20]. For these reasons, BPH is not currently considered a precursor of PCa or a premalignant lesion.

Prostatic intraepithelial neoplasia (PIN) is defined by a “neoplastic growth of epithelial cells within preexisting benign prostatic acini or ducts” [21]. When PIN develops, epithelial cells from acini or ducts of the prostate become abnormal, yet without functional disruption. According to the histological grading of malignant cells, PIN may be subdivided in low- and high-grade (HGPIN) categories, being the latter considered a percursor of PCa with an estimated 10-year lag [22]. HGPIN and PCa share a variety of features, mainly concerning (1) statistical data, with 73% of prostate glands that harbor cancer having HGPIN lesions, compared to 32% of healthy glands [23]; (2) molecular alterations such as loss of chromosome 8p, gain of 8q or harboring of TMPRSS2-ERG fusion gene [22]; and (3) the partial lack of a basal cell layer in HGPIN, which further validates HGPIN as a precursor lesion due to the absence of basal cells in PCa, suggesting a progressive loss of this feature [22]. Although not all prostate tumors necessarily result from the development of a HGPIN lesion, the

degree of similarities between HGPIN and PCa suggests a causal relationship. Therefore, HGPIN currently remains the most well-established and likely percursor of PCa.

Prostate adenocarcinoma arises as the most common malignant disorder in the prostate, being an extensively heterogeneous pathology which can lead to very different outcomes, from clinically indolent to extremely aggressive ones. Prostate adenocarcinoma accounts for about 95% of prostate cancers, hence being usually designated by 'prostate cancer' or PCa as a matter of simplification. The majority of prostate cancers are acinar adenocarcinomas, while other tumor categories such as ductal or mucinous adenocarcinoma are extremely rare [24]. Considering worldwide cancer statistics in males, PCa is currently the second most incident cancer in males (Figure 2) but only the fifth leading cause of death from cancer (Figure 3), with an estimated 1.1 million new cases and 307,000 deaths in 2012 [25]. In Portugal 6,622 new cases of PCa were diagnosed in 2012, being the most incident cancer in males and the third most lethal cancer with 1,582 registered deaths [25]. These numbers clearly demonstrate that PCa is a relevant health concern nowadays.

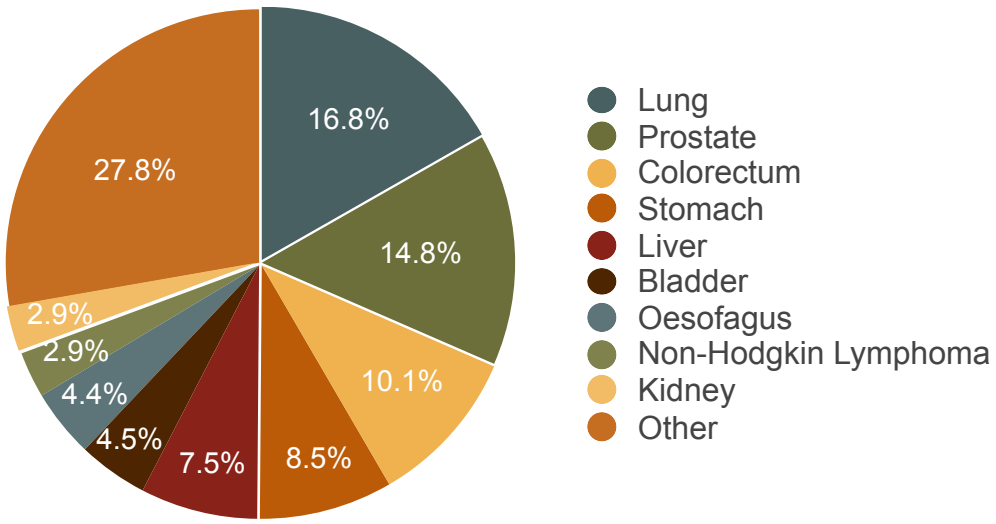


Figure 2: Cancer incidence in males worldwide [25].

In spite of being a very common and widespread disease, there are only three well-characterized risk factors for PCa: age, ethnicity and family history [26]. All of them are inevitable and do not directly depend on any human behavior, which restricts the possibility of a widespread action on disease prevention. Age is the most relevant risk factor: the risk of having PCa is negligible until age 50 but rises after age 55 and reaches a peak at 70-74 years [27]. Ethnicity also plays an important role, with African American (AA) patients having higher risk than their counterparts, with more than

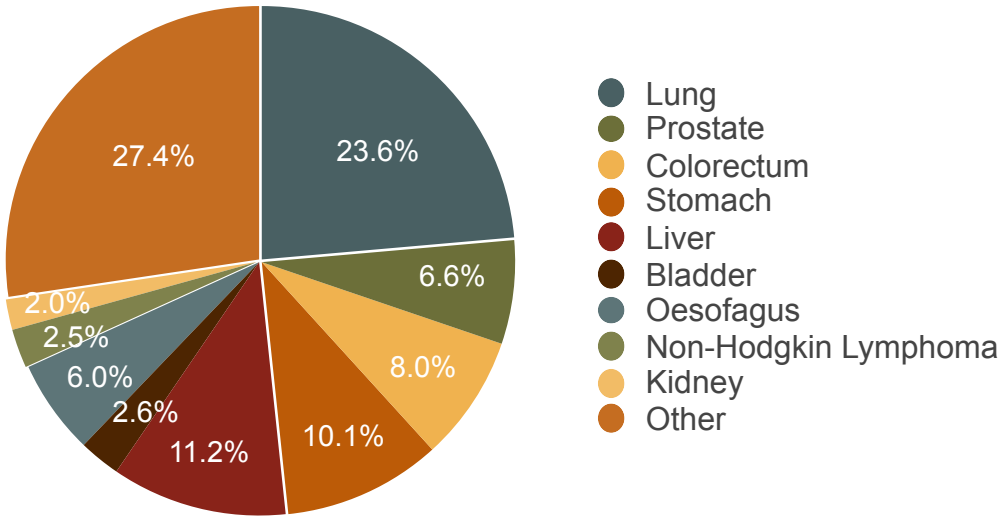


Figure 3: Cancer mortality in males worldwide [25].

twice of the mortality rate compared to Caucasian Americans [28]. Reasons for this phenomena are still widely unknown, although chromosome 8q24, which is one of the most commonly amplified chromosomal regions may, at least partially, explain the higher incidence in AA men [29]. Family history also increases the risk of developing the disease: a brother or father with PCa at least doubles the risk of a man having PCa [27] and it may increase until 17 times depending on the number of affected first-line relatives [30]. About 9% of diagnosed men have hereditary PCa, which anticipates the onset for 6-7 years [26], but only the presence of a germline BRCA2 mutation has shown an increased risk of early and aggressive disease [31, 32]. Additional risk factors such as diet [33] or metabolic syndrome [34] have been proposed, however without sufficiently clear evidence.

1.3. Screening, Diagnosis and Prognosis of Prostate Cancer

The goal of PCa screening is to detect cancer before symptoms arise. The two most widely screening tools are prostate-specific antigen (PSA) level in serum and digital rectal examination (DRE), an examination in which a physician directly assesses prostatic abnormalities by finger examination of the prostate gland through the rectum.

PSA is a serine protease produced by prostatic epithelial cells whose function is to cleave semenogelins from the gel matrix of the seminal coagulum, allowing sperm to move freely [35]. PSA test was approved in 1986 for monitoring disease progression and in 1994 (together with DRE) for screening asymptomatic man. Two large randomized studies have shown that PSA screening increased the diagnosis

rate particularly at early stages of cancer development, but the existence of a benefit in overall survival or cancer-specific survival is unclear [36, 37]. These observations have generated controversy regarding the use of PSA test because it could be responsible for the detection of non life-threatening tumors (overdiagnosis) and subsequent unnecessary treatment (overtreatment) [38]. Furthermore, PSA is prostate-specific but not cancer-specific and other prostatic pathologies such as prostatitis or BPH may increase PSA levels leading to false-positive results. In fact, PSA-based screening represents a high economic burden for PCa: 1410 men are needed to be screening and 48 PCa cases to be treated in order to prevent one disease-related death [37]. Therefore, PSA test remains a useful screening tool but widespread screening is not recommended and its use should be limited to men expressing preference for screening [26, 39].

According to present guidelines, men with serum PSA higher than 4 ng/mL and/or abnormal result from DRE must perform a prostate biopsy. The transrectal ultrasound (TRUS)-guided biopsy is the gold-standard method for the obtainment of prostate tissue samples for histological studies. 10 to 12 cores should be obtained from different parts of the prostate gland and additional cores may be obtained from suspicious areas [26]. Definite diagnosis is not limited to PSA level or DRE result and always depends on the histological confirmation of a cancerous lesion by prostate biopsy.

In order to determine the prognosis of PCa and the most appropriate therapeutic option for each patient, tumor staging and grading emerge as the two most valuable parameters regarding PCa characterization.

PCa staging is an indicator of how far the cancer has spread. Given the importance of knowing cancer extension to successfully evaluate prognosis and plan the treatment, efforts have been made in order to find a standardized staging system for clinical application [40]. The TNM system is currently the most broadly used staging tool and it evaluates three different components: the local staging, which is the extension of primary tumor (T); the involvement of lymphatic nodes (N); and the presence of metastases (M). According to the timing and source of data collection, PCa staging may be subdivided in (1) clinical stage (cT), which is the information on cancer extension that is collected prior to treatment onset; and (2) pathological stage (pT), which is determined after direct macroscopic and microscopic examination of the surgical specimen and thus only obtained after radical prostatectomy. Pre-treatment local staging (T) is based on DRE findings and possibly MRI and it is obtained for all patients, while lymph-node staging (N), which is preferentially measured by nodal

evaluation, or metastatic staging (M), achieved by bone scan, are only performed in high-risk patients [41]. A summary of the used criteria for tumor staging according to TNM system can be seen below in Table 1.

PCa grading consists of the measurement of the microscopic features of cancer cells to estimate the aggressiveness of the tumor. Named after Donald F. Gleason's proposal in 1966, the Gleason grading system is the most widely used tool for PCa grading. Cancer cells are classified according to their differentiation status from Grade 1 (most differentiated) to Grade 5 (least differentiated) and Gleason Score (GS) is calculated by the sum of the grade of the most extensive component with the sum of the highest grade regardless of its extent. Consequently, GS may vary between 2 (1+1) and 10 (5+5) [41]. The concept of Grade Group (GG) was recently proposed in an attempt to more accurately simplify the high GS number for clinically irrelevant tumors and to distinguish important differences between GS 7 (3+4) and GS 7 (4+3) tumors [42]. Therefore GG 1 to 5 account for GS ≤ 6, GS = 7 (3+4), GS = 7 (4+3), GS = 8 and GS = 9, respectively and these prognostically distinct groups must be clinically used in conjunction with the previous Gleason Score concept [26, 43], as illustrated in Figure 4.

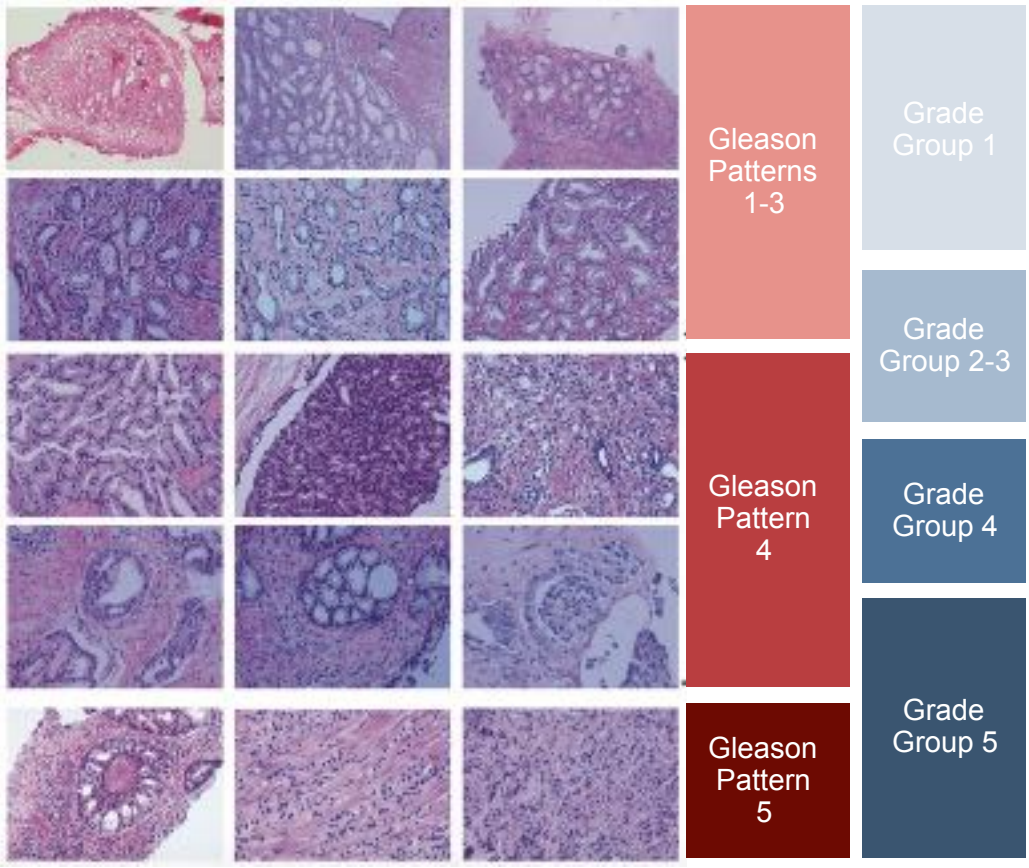


Figure 4: H&E-stained sections illustrating the different Gleason patterns and corresponding Grade Groups (adapted from [43]).

Table 1: TNM classification for PCa by the UICC/AJCC (adapted from [40]).

	Clinical Stage	
Tx	Primary tumor cannot be assessed	
T0	No evidence of primary tumor	
T1	Clinically inapparent tumor that is not palpable	
	T1a	Tumor incidental histologic finding in 5% or less of tissue resected
	T1b	Tumor incidental histologic finding in more than 5% of tissue resected
	T1c	Tumor identified by needle biopsy found in one or both sides, but not palpable
T2	Tumor is palpable and confined within prostate	
	T2a	Tumor involves one-half of one side or less
	T2b	Tumor involves more than one-half of one side but not both sides
	T2c	Tumor involves both sides
T3	Extraprostatic tumor that is not fixed or does not invade adjacent structures	
	T3a	Extraprostatic extension (unilateral or bilateral)
	T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall	
	Pathological Stage	
T2	Organ confined	
T3	Extraprostatic extension	
	T3a	Extraprostatic extension (unilateral or bilateral) or microscopic invasion of bladder neck
	T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall	
Nx	Regional lymph nodes were not assessed	
N0	No positive regional lymph nodes	
N1	Metastases in regional lymph node(s)	
M0	No distant metastasis	
M1	Distant metastasis	
	M1a	Nonregional lymph node(s)
	M1b	Bone(s)
	M1c	Other site(s) with or without bone disease

Considering the referred classification systems for tumor staging, three risk groups with different expected outcomes have been defined for patients diagnosed with PCa [26]. Low-risk PCa harbors organ-confined tumors with PSA < 10 ng/mL, GS < 7 and cT 1-2a. Intermediate-risk PCa accounts for localized tumors with at least one of the following conditions: PSA 10-20 ng/mL, GS 7 or cT 2b. High-risk PCa englobes locally advanced tumors with any PSA level or GS and with cT 3-4 or cN+ as well as organ-confined tumors with at least one of the following conditions: PSA > 20 ng/mL, GS > 7 or cT 2c [26]. The risk group constitutes an extra tool for the physician to choose one of the available therapeutic options, however other important aspects such as life expectancy, comorbidities or therapeutic side effects must be taken into account in order to further perform a personalized therapeutic approach.

1.4. Therapeutic Options for Prostate Cancer

Considering that (1) PCa frequently affects elderly man; (2) about 80% of prostate tumors are presumably detected in an early, organ-confined stage [44] and (3) patients with low-risk PCa present high 15- and 20-year survival rates [45, 46], one of the main challenges regarding PCa treatment nowadays is the prevention of overtreatment of clinically irrelevant tumors that are very unlikely to kill the patient. Active surveillance (AS) emerges a strategy where low-risk patients are not treated but instead kept under surveillance through systematic DRE and PSA tests and eventually rebiopsies. Patients under AS harbor tumors that are characterized by a very slow growth. This approach allows them to be spared from adverse effects of radical therapy and still benefit from therapy with curative intent in case of cancer progression [47]. This approach is currently limited to patients within tumor-confined stages (cT2 or less), GS equal or less than 6, serum PSA levels below 10 ng/mL and more than 10 years of life expectancy [26]. Some studies have shown a high 10-year disease-specific survival for AS patients with levels comparable to those who performed radical therapies [48, 49]. Watchful waiting (WW) is a similar procedure in which patients with a life expectancy inferior to 10 years who are not candidates for radical therapy are closely followed and noncurative treatment is applied only in case of development of symptoms. This approach is more frequent among very old patients (since it is very unlikely that the tumor kills the patient due to his advanced age) and its main goal is to minimize treatment-related toxicity and side effects. It has been reported that there was no difference in 10-year overall survival and cancer-specific survival for low-risk patients when comparing WW and radical surgery, which thereby supports this novel therapeutic approach [50].

Among non-conservative therapeutic possibilities, radical prostatectomy (RP) arises as one of the most common procedures and its purpose is the complete surgical removal of the prostate gland and seminal vesicles without compromising the normal functioning of urinary and, when possible, the reproductive system. RP is limited to patients with organ-confined disease, PSA values below 20 ng/mL and life expectancy of more than 10 years [26]. RP may be performed in low-, intermediate- and high-risk patients, however extraprostatic extension and GS > 7 are common contraindications for the latter group. Complementary therapies such as extended nodal dissection are frequently performed particularly in high-risk patients. RP has shown a benefit in overall and disease-specific survival for intermediate-risk patients compared with WW [51], although side effects such as postoperative incontinence or erectile dysfunction may arise. External-beam radiotherapy (EBRT), a therapeutic option in which tumor is eradicated through external emission of ionizing radiation, and brachytherapy, where small radiation-emitting beads are implanted nearby the tumor leading to an internal emission of radiation are also curative treatment choices with benefits similar to RP [52].

Despite the high proportion of PCa cases within a localized tumor stage at time of diagnosis, about 20% of patients are diagnosed within an advanced stage. Due to the almost ubiquitous expression of AR in PCa cells and their dependence on androgens for growth and proliferation, virtually all prostate tumors are androgen-responsive at least on their early stages [53]. Based on this principle, androgen deprivation therapy (ADT) accounts for the suppression of androgen production and remains the standard procedure for non-localized PCa, being also frequently used as an adjuvant therapy after curative treatment and also as a neo-adjuvant option before primary treatment (particularly among patients with higher GS at time of biopsy) as well as a second-line therapy in patients who developed a cancer relapse after initial treatment. The two main methods for androgen deprivation are (1) surgical castration, involving a bilateral orchidectomy (surgical excision of the testicles), which quickly and effectively reduces testosterone levels in circulation [54] but leads to a strong decrease in quality of life and patient regretfulness due to its irreversible nature [55, 56]; and (2) medical castration, where decreased androgen levels are caused by the administration of drugs that manipulate the hypothalamic–pituitary–gonadal axis, which is responsible for hormone production [56]. Although strong improvements on ADT have been achieved in the last years, eventually all tumors evolve into a castration-resistant prostate cancer (CRPC) stage, in which cancer is able to overcome the androgen-dependence and maintain its growth by activating alternative

proliferation pathways. CRPC is the final and incurable stage of PCa and inevitably leads to patient death. On average, it arises only 18 months after hormone treatment [57]. At this point, docetaxel-based chemotherapy is a common therapeutic option but with an estimated survival benefit of merely 2 months [58]. Most of CRPC patients present painful metastases and their treatment is restricted to palliative options.

1.5. The Arising of a Biochemical Recurrence

When a patient is submitted to a radical therapeutic option such as RP or EBRT, cancer cells are thought to have been surgically removed or effectively killed through irradiation, respectively. However, it is estimated that 35% of patients that underwent RP and 30% of patients who performed EBRT will develop a biochemical recurrence (BCR) within 10 years [59, 60]. Since both therapeutic options are limited to patients with presumably organ-confined disease, it is reasonable to assume that BCR happens because undetectable subpopulations of cancer cells have spread beyond the prostatic gland and managed to escape primary treatment and survive.

After performance of RP, PSA value is expected to fall to undetected levels, since the prostate has been removed, whereas EBRT-treated patients may have a low but detectable postoperative PSA, which is called PSA *nadir*. It is currently accepted that a PSA level > 0.2 ng/mL upon two consecutive increases after RP represents a BCR [61], while BCR after EBRT is represented by a PSA value of 2 ng/mL above PSA nadir [62]. Postoperative PSA test is considered an appropriate tool to monitor the occurrence of BCR after treatment. In fact, similarly to all prostatic cells, cancer cell populations which survived curative therapy produce PSA and may, at some point, proliferate and generate detectable levels of PSA in serum. Nevertheless we should have in mind that PSA is prostate-specific but not cancer-specific and thus a detectable PSA level should not be seen as a certainty of the presence of cancer cell populations, since it may reflect, for instance, the presence of residual benign tissue [63].

In patients with BCR, less therapeutic options are available. Salvage radiotherapy is a common and effective therapeutic choice with post-therapy PSA values falling to undetectable levels in about 60% of patients [64]. ADT also emerges as a possible salvage treatment, however its use as a second-line therapy after RP must not be offered to asymptomatic patients with BCR or patients with high PSA doubling times and thus very low progression rates [26].

Although PSA is a commonly used tool during patient follow-up after treatment, its ability to predict the occurrence of a BCR before therapy is very limited. In fact, clinicopathological features such as Gleason score, surgical margin status or pathological stage have only shown a limited association with BCR-free survival time [65]. Therefore, recalling that BCR may arise due to the existence of micrometastatic cancer cells spread beyond the prostatic gland, a biomarker that could detect the presence of those cancer cell subpopulations before the performance of RP is needed in order to determine if BCR is likely to happen and thus RP and/or adjuvant treatments are needed. Monitoring cancer cells detachment and invasion at a molecular level would certainly allow a more effective treatment choice and reduce patient anxiety, the number of postoperative relapses and consequently the need for further treatment with inevitably associated side effects.

2. MicroRNAs

2.1. The World of non-coding RNA

Enunciated by Francis Crick in 1958, the “central dogma of molecular biology” firstly proposed that biological information is uniquely stored in the nucleotide sequence of DNA and flows unidirectionally from DNA to RNA and from RNA to protein [66]. Although some adjustments to this theory were posteriorly performed, highlighting that a backward flux of information from RNA to DNA was equally possible mainly due to published works reporting the use of viral RNA as a template for DNA synthesis in the context of infection and transformation by viruses [67], RNA was initially seen as a merely transient form of information between DNA and protein. However, protein-coding DNA is estimated to constitute less than 2% of the human genome [68] contrasting with the fact that almost all genome is transcribed at some level [69], which brought into question the role of RNA transcripts resulting from non-protein-coding DNA (previously called *junk* DNA due to its apparent uselessness).

These different forms of RNA, generally referred to as “non-coding RNA”, include not only the well-characterized ribosomal RNA and transfer RNA but also new recently found classes of RNAs such as snRNAs, snoRNAs, circular RNAs and — of particular interest for this work — microRNAs. Despite their general low expression levels [70], the functionality of many of these forms of non-coding RNA has been progressively demonstrated, inevitably challenging the way non-coding RNAs are seen in context of gene expression within the cell [71].

2.2. MicroRNAs: Biogenesis and Functionality

MicroRNAs (miRNAs; miR) are very small non-coding RNA molecules with an average of only 22 nucleotides. They are able to suppress gene expression at the level of translation by directly interacting with (and thereby silencing) molecules of mRNA [72]. Firstly discovered in 1993 through developmental studies on nematode *Caenorhabditis elegans*, miRNAs have been shown to exert their action by directly matching the 3’-untranslated regions (UTRs) located at the end of their mRNA targets [73, 74]. In the last 20 years, knowledge on miRNA field has been exponentially expanding, particularly concerning miRNA biogenesis, their *modus operandi* and their role whether during normal homeostasis or the development of several pathologies.

One of the most relevant aspects of miRNA biology is their promiscuous association with mRNA molecules: a single miRNA may regulate dozens of different transcripts whereas the same mRNA molecule may be targeted by several different miRNAs [75, 76]. Accordingly, miRNAs have been virtually linked to all normal and pathological processes, being estimated that up to 60% of protein-coding gene expression may be regulated by miRNA activity [72]. Moreover, miRNA expression has also been shown to be extensively tissue-specific allowing the identification of specific miRNA expression profiles for each cell type. Although putative functions of individual miRNAs have progressively been unveiled mainly through ectopic expression studies, loss-of-function studies carrying individual inactivation of miRNAs have rarely shown to dramatically change gene expression levels and to have strong phenotypic consequences. This suggests that miRNAs may act not as strong expression repressors but rather as gene expression fine-tuners that guarantee rapid and cell-dependent changes in protein synthesis without altering upstream transcription process [77].

There are currently 1,881 *homo sapiens* miRNA sequences that have been identified and published on miRBase, a widely used online miRNA repository [78]. However, due to the small size of miRNAs, it is quite difficult to assure that a certain sequence is indeed a miRNA and it has been proposed that many of these putative miRNAs are false positives considering only 523 sequences to be *bona fide* miRNA genes [79]. Between 50-80% of miRNA genes are thought to be localized at intronic regions (i.e., segments of DNA that do not code for protein) within host genes [80, 81] and their transcription results from further processing of the removed introns as part of the normal host gene transcription. Alternatively, miRNA genes can be found in intergenic regions and transcribed as individual units or gene-independent clusters containing several different miRNAs [82].

A summary of the most relevant steps of miRNA biogenesis can be seen in Figure 5 [83]. Transcription of miRNA genes is performed by RNA polymerase II, generating transcripts named pri-miRNAs with variable length (usually between 1000-3000 nucleotides) that harbor a region of imperfect double-strand complementarity, called a stem-loop structure, where the future mature miRNA is contained [84]. This region is recognized by the ‘human microprocessor’, a protein complex formed by DGCR8 protein that binds to the pri-miRNA and ribonuclease Drosha which further processes pri-miRNAs by enzymatic cleavage, generating a 65-nucleotide-long hairpin-shaped precursor-miRNA (pre-miRNA) with two arms attached by a loop [85]. An alternative mechanism to this canonical pathway has also been reported: due to their small size, miRNAs arising from very short introns — referred to

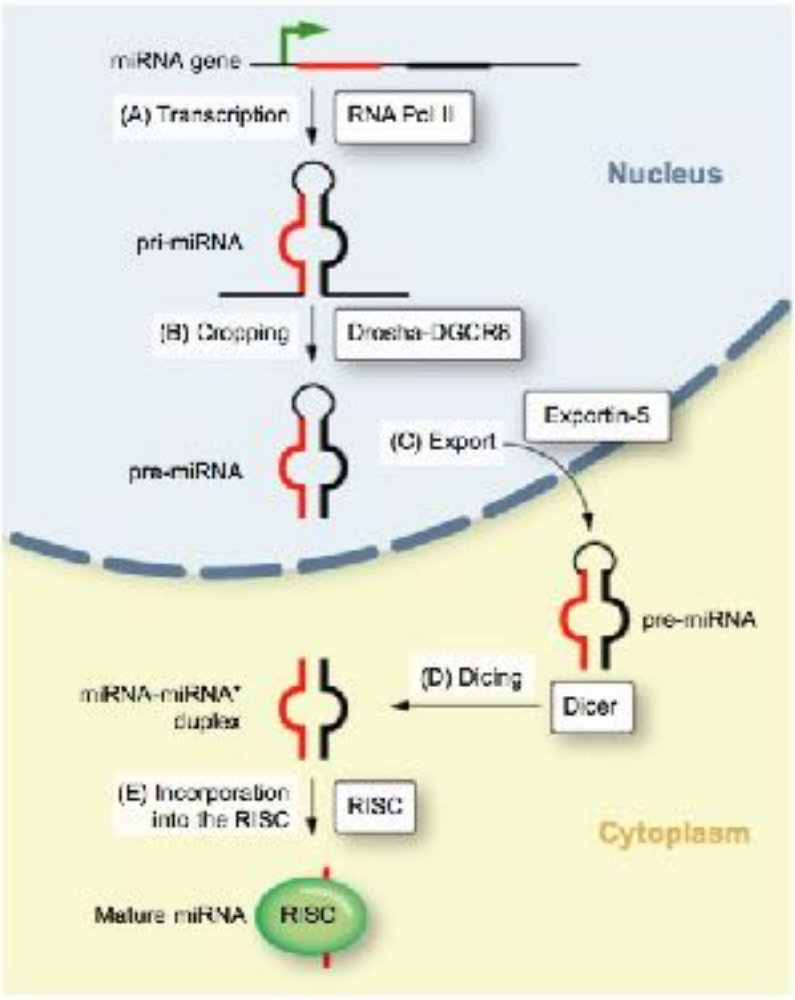


Figure 5: The process of miRNA biogenesis (adapted from [83]).

as mirtrons — are processed by the normal splicing machinery and at this point its structure already mimics the pre-miRNA structure, therefore being able to bypass Drosha-DGCR8 cleavage [86].

All previously described steps take place within cell nucleus. Thereafter, pre-miRNA is transported to the cytoplasm via Exportin 5 (XPO5) and further cleaved by ribonuclease Dicer, which together with transactivator-RNA binding protein (TRBP1) removes the loop portion, giving rise to a duplex formed by two short and partially complementary RNA strands [87]. This duplex is composed of a guide strand holding the mature miRNA and a passenger strand which contains the miRNA*, a species that is usually degraded and thus much less abundant than the correspondent miRNA — for instance, miR-182 and miR-182* share the same pre-miRNA but miR-182 is much more extensively expressed. However, it is noteworthy that both arms may have similar expression levels and therefore should be nominally distinguished with a -5p suffix and a -3p suffix for guide and passenger strands respectively — for instance, miR-28-5p and miR-28-3p share the same pre-miRNA and both are expressed [88].

The mature miRNA is then transferred to Argonaute proteins via TRBP1 assembling the RNA-induced silencing complex (RISC), a protein complex that effectively regulates gene expression by enabling miRNA-mRNA matching and subsequent inhibition of mRNA translation [82]. Tridimensional structure of RISC complex allows the exposure of miRNA *seed sequence*, the portion of miRNA comprised between nucleotides 2-8 that effectively matches the 3'UTR of mRNA [89]. The degree of miRNA-mRNA complementary determines the outcome of miRNA regulation: a perfect or near-perfect match between miRNA and mRNA (above 95% of complementarity) target induces mRNA cleavage and subsequent destruction, although this is considered a very rare phenomenon in mammals; contrarily, an imperfect complementarity (below 95%) leads to a translation repression [90]. Notably, some miRNAs have been described as translation activators instead of repressors [76], although this surprising regulation phenomena remains an exception and not the rule.

2.3. MicroRNAs as Cancer Biomarkers

Following the expectations of miRNAs to be linked to virtually all biochemical processes including disease development, researchers have focused on exploiting the association between miRNAs and different steps of carcinogenesis during the last 15 years. In 2002 miRNAs were firstly described to be differentially expressed in cancer by Calin *et al*. who demonstrated that miR-15a & miR-16-1 cluster was downregulated in B cells of patients suffering from chronic lymphocytic leukemia [91]. Subsequent studies on different cancer types were published suggesting a global and consistent miRNA deregulation in cancer [92, 93]. Since miRNAs promote a repression on gene expression, miRNAs that target mRNAs encoding oncogenes (*i.e.*, genes with potential of promoting cancer development) are classified as tumor suppressor miRNAs, while miRNAs inhibiting the expression of tumor suppressor genes are defined as oncogenic miRNAs or oncomiRs [94]. Therefore, a global deregulation of miRNA profile involving loss of tumor supressor miRNAs and gain of oncogenic miRNAs is thought to be closely related with cancer development.

Putative mechanisms of miRNA loss and gain are thought to be similar to the ones that drive repression of tumor suppressor genes and activation of oncogenes [94]. Loss and gain of miRNA expression has been associated not only with genetic alterations such as chromosomal deletions [91], amplifications or mutations [95] but also due to epigenetic mechanisms such as promoter hypermethylation [96] or histone

modifications [97]. This hypothesis is further sustained by studies reporting that miRNA genes are frequently located within fragile genomic regions that usually give rise to cancer-related aberrant genomic alterations [98].

Since miRNA expression signatures differ between cancer and normal tissue and also between cancer subtypes in a very consistent way, miRNAs have progressively emerged as stable cancer-specific biomarkers that can help to determine diagnosis, prognosis, treatment response and other clinical parameters of patients with cancer. Importantly, in 2008 miRNAs have been found to circulate in biological fluids, such as plasma or serum, with a remarkable stability [99], thereby broadening their potential both as tumor-confined and fluid-circulating biomarkers. However, it is fundamental to clarify the origin and putative biological role of a plasma-circulating miRNA in a patient diagnosed with cancer. As recently reviewed, cancer cells or cells from tumor microenvironment may actively release tumor-promoting miRNAs into blood circulation, but healthy blood cells such as peripheral blood mononuclear cells (PBMC) might also secrete tumor-suppressive miRNAs with as part of host response against cancer [100], and thus a cautionary approach should be performed regarding the potential of miRNAs as circulating biomarkers in cancer.

2.4. Prostate Cancer: Which Role for microRNAs?

Being one of the major concerns in the context of malignant diseases due to its very high incidence among male population, efforts have been made in order to investigate how to achieve early diagnosis and estimate prognosis with higher accuracy. Considering the great potential of miRNAs as putative cancer biomarkers, numerous studies have contributed to the identification of the most relevant miRNAs in PCa biology and the establishment of a PCa-specific miRNA expression profile [101-105]. For the purposes of this work, a literature review was conducted with the goal of assembling the currently knowns and unknowns about the deregulation and biological role of miR-182 and miR-375 in the context of PCa.

2.4.1. miR-182

miR-182 belongs to the polycistronic miR-183/96/182 cluster located on human chromosome 7q32.2 [106]. Members of this cluster are expressed at high levels in most cancers and generally considered oncomiRs. Its upregulation in PCa compared to healthy prostatic tissue is strongly described. miR-182 has been reported to be upregulated in prostate tumors compared to healthy tissue in a genome-wide expression study [107], microarray analysis [108] with further qRT-PCR validation

[109], TCGA dataset [110], GEO dataset [111] and in prostate tumor xenograft tissues [112]. It is also overexpressed in DU145 cell line [113]. miR-182 is also highly expressed in tumors with higher Gleason grades [114]. Overexpression in metastatic vs localized PCa was also reported in GEO dataset [115].

As a biomarker, miR-182 expression in cancer tissue has been shown to improve diagnosis [116] and predict disease progression after RP [117]. miR-182 expression was recently found to be packaged in exosomes broadening their potential as noninvasive biomarkers [118]. However, to the best of our knowledge no evidence of miR-182 differential expression in biofluids such as plasma in the context of PCa has been published.

Regarding miR-182 biological role, miR-96/182/183 cluster may be involved in suppression of zinc transporters and thus may play an oncogenic role since zinc is thought to have a protective role against PCa [108]. Importantly, miR-182 have been shown to promote cell invasion and proliferation by directly targeting FOXF2, RECK, MTSS1 [119], FOXO1 [120] and ARRDC3 [121] and also in PC3 cells by targeting NDRG1 [122]. miR-182 may support angiogenesis through inhibition of PDH2 and FIH1, which are negative regulators of hypoxia-inducible factor 1α (HIF-1α) [123]. However, miR-182 has shown to reduce tumor invasion through inhibition of invasion-promoting protein GNA13 [124] and to simultaneously inducing tumor growth self-sufficiency and mesenchymal-epithelial transition (MET) through SNAI2 targeting [125]. miR-182 also inhibited cell proliferation on statin-treated [126] (targeting BCL2) and mangiferin-treated [127] prostate cell lines.

2.4.2. miR-375

As one of the most consistently described miRNAs in PCa, miR-375 offers great potential as a diagnostic and prognostic biomarker. Overexpression of miR-375 in primary PCa tissues *vs* matched normal prostatic tissue (MNPT) has been reported in several studies [101, 103, 109, 128-131], suggesting an intra-tumoral oncogenic role for miR-375 in PCa. miR-375 is included in a 3-miR panel able to identify 80% of patients (AUC = 0.88) [109] and in a 6-miR panel able to identify 70.8% of patients (AUC = 0.854) [129] thus potentially having diagnostic value. Although a correlation between miR-375 and clinicopathological variables was not found in three of the aforementioned studies [109, 128, 129], Selth and colleagues observed that intra-tumoral miR-375 levels predict time to BCR after RP independently of GS and clinical stage [103].

Similarly, miR-375 shows a noteworthy potential as a circulating biomarker. Increased levels of miR-375 in serum of PCa patients have been consistently detected [101, 103-105, 130, 132], despite a notable exception described by Kachakova *et al.* who found circulating miR-375 to be downregulated in PCa compared to BPH controls [133]. Interestingly, overexpression of miR-375 has been mainly reported in advanced-stage PCa patients, particularly: metastatic CRPC patients *vs* healthy men [103, 104], CRPC *vs* low-risk localized PCa [130], disseminated PCa (N1/M1 and CRPC) *vs* both localized PCa and BPH controls [105] and metastatic vs localized PCa [101], which suggests that miR-375 role may have a particular focus on disease progression into more advanced stages. Upregulation of serum miR-375 was also found in localized PCa compared to both BPH and healthy controls [132], which is in agreement with the described miR-375 oncogenic role. Serum expression of miR-375 in combination with expression of its target protein suPAR is associated with lymph-node positivity [101] and with high tumor stages (T3/ T4 vs T1/T2) [132] and correlated with poor overall survival and BCR-free survival [132], thus reinforcing the prognostic value of miR-375. Studies on plasma-circulating microvesicles and exosomes have reported that exosomal miR-375 is overexpressed in metastatic *vs* non-metastatic PCa [134] and negatively associated with overall survival in CRPC patients [135]. miR-375 upregulation in PCa has also been described in other biofluids such as urine [136], although still further evidence is still needed.

Although the potential of miR-375 as a biomarker appears to be a common characteristic in PCa, its specific biologic role in tumor development is still unclear. miR-375 was reported to be a direct target of PHLPP1, a phosphatase and potent inhibitor of proliferative signaling pathways [131]; Sec23A, a protein transport mediator which was able to reduce growth properties of PCa cells in vitro [128]; and CBX7, a member of the Polycomb repressive complex I that was found to be downregulated in advanced PCa [137]. miR-375 also shows a positive association with AR: Chu *et al.* observed that AR-positive cell lines (22Rv1, C4-2, LNCaP) present higher expression levels and hypomethylation of miR-375, while AR-negative cell lines (DU145, PC-3) show the opposite profile. They suggested that AR promotes miR-375 higher expression via promoter demethylation [138]. DHT-stimulated LNCaP cells release miR-375 to the incubation medium, further suggesting that miR-375 could be androgen-sensitive [139]. However, ectopic expression of miR-375 decreased invasion and migration in PC3, DU-145 and C4-2B cell lines and facilitated epithelial differentiation, intriguingly acting as a tumor supressor through a ZEB-1/miR-375/ YAP-1 pathway that regulates EMT in PCa cells [140]. Our research group recently

suggested a dual role for miR-375 in prostate carcinogenesis, acting both as an oncomiR in 22Rv1 cells and as a tumor suppressor miR in PC-3 cells [141]. Even though a potential dual role of miR-375 in different prostate tumors is a possible scenario, its biological role is still unclear and thus further research is still required.

Aim of this Study

Notwithstanding the substantially high worldwide incidence of PCa, most of the prostatic tumors are diagnosed at early stages, in which patients may benefit from radical curative therapy such as RP. However, a considerable proportion of patients submitted to RP still develop late events such as recurrence, castration-resistance and metastasis, even though their primary tumor had been surgically removed. Although several studies have reported differentially expressed miRNAs in PCa, that may act as potential diagnostic or prognostic biomarkers, little is known about changes in miRNA levels before and after radical therapy and their putative relevance as biomarkers of postoperative cancer-related late events. Therefore, we hypothesized that plasma-circulating miRNA levels might be able to discriminate patients that develop late events from patients that do not.

For the purposes of this work the following objectives were drawn:

1. To assess miRNA levels in PCa and non-neoplastic tissue using the publicly available TCGA dataset;
2. To confirm miRNA deregulation in tumorous tissues from patients included in the validation cohort;
3. To determine miRNA levels in preoperative and postoperative plasma from patients included in the study cohort;
4. To identify associations between miRNA expression levels and PCa patients' clinicopathological features.

Material and Methods

1. The Cancer Genome Atlas dataset

miRNA expression levels of 326 PCa and 50 non-neoplastic tissues were retrieved by in The Cancer Genome Atlas (TCGA) dataset and analysed by collaborators from Instituto de Investigação em Ciências da Vida e Saúde (ICVS) of the University of Minho (Braga, Portugal).

2. Patients and sample collection

A total of 210 patients harboring PCa that were submitted to RP at Portuguese Oncology Institute of Porto (Porto, Portugal) between 2001 and 2012, were recruited for this study. Peripheral blood was collected into EDTA-containing tubes at two distinct time points: immediately before RP (1 day prior to surgery) and during routine patient follow-up, after treatment. Plasma was obtained from blood by centrifugation (2,000 rpm; 10 min; 4ºC) and subsequently stored at -80ºC until further use. Prostate specimens from a subset of 100 patients were frozen after surgical procedure and stored at -80ºC. After the presence of tumor was confirmed by hematoxylin-eosin staining procedure, histological slides from frozen tissue fragments were cut using a cryostat and posteriorly stored at -80ºC until further use. Healthy prostate specimens from a group of 15 patients who performed cystoprostatectomy due to bladder cancer were used as negative controls after confirmation of the absence of malignant prostatic tissue.

This study was approved by the institutional review board [Comissão de Ética para a Saúde-(IRB-CES-IPOFG-EPE 120/015)] of Portuguese Oncology Institute of Porto, Portugal. Informed consent was obtained from all patients according to institutional regulations.

3. RNA extraction from Tissue

RNA from PCa tissue was isolated using Triple Xtractor Reagent (GRiSP, Porto, Portugal). Briefly, 1 mL of reagent was added to tissue slides with subsequent tissue homogenization using a Cordless Pestle Motor and Disposable Pestles (VWR, Radnor, PA, USA). 200 µL of chloroform were added and the mixture was thoroughly vortexed for 15 seconds. Samples were centrifuged (10,600 rpm; 15 min; 4ºC) using

Centrifuge 5430R (Eppendorf AG, Hamburg, Germany) for phase separation and RNA-containing upper aquous phase was mixed with 500 µL of isopropanol. After 10 min of incubation samples were centrifuged (10,600 rpm; 10 min; 4ºC) for RNA precipitation and two consecutive wash-and-centrifugation cycles with 75% ethanol (8,400 rpm, 5 min, 4ºC) were performed. Supernatant was removed and remaining *pellet was* air-dried for 30 min. Resuspension with 30 µL of RNase-free water was performed following 30 min of incubation on ice. Tissue RNA concentration and purity were subsequently measured using Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies).

4. RNA extraction from Plasma

Circulating RNA extraction was obtained using miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany). Briefly, 1 mL of QIAzol Lysis Reagent (Qiagen) was added to 200 µL of plasma to denature protein complexes and RNases. 200 µL of chloroform were added and samples were centrifuged (12,000g; 15 min; 4ºC) using Centrifuge 5430R (Eppendorf AG) to perform phase separation. 900 µL of ethanol were added to 600 µL of RNA-containing upper aqueous phase and thoroughly mixed. Washes and subsequent centrifugations were performed on RNeasy MinElute spin columns with RWT Buffer (10,000 rpm; 15 seg; 25ºC), RPE Buffer (10,000 rpm; 15 seg; 25ºC) and 80% ethanol (10,000 rpm; 2 min; 25ºC). Columns were centrifuged with opened lids (13,000 rpm; 5 min; 25ºC) in order to dry the membrane. 14 mL of RNase-free water were added to the membrane and samples were centrifuged (13,000 rpm; 1 min; 25ºC)to elute RNA. RNA concentration and purity were posteriorly determined using Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies).

5. cDNA synthesis

microRNA-specific cDNA synthesis was performed using TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), following manufacturer's instructions. Briefly, 100 ng of RNA in a volume of 5 µL were transferred to each well of a GRS 96w PCR plate (GRiSP) and the following mix was added per well: 0.15 µL of 100mM dNTPs; 1 µL of MultiScribe™ Reverse Transcriptase; 1.5 µL of 10x Reverse Transcription Buffer; 0.19 µL of 20 U/µL RNase inhibitor; and 4.16 µL of DNase/RNase-Free Water (GIBCO, Grand Island, NY, USA). 3 µL of 5x TaqMan microRNA looped RT primer (Applied Biosystems) were added to each well and reverse transcription was performed with Veriti® Thermal Cycler (Applied Biosystems) within the following conditions: 30 min at 16ºC; 30 min at 42ºC; and 5 min at 85ºC (Figure 6). A 1:2 dilution with DNase/RNase-Free Water (GIBCO) was performed after cDNA synthesis.

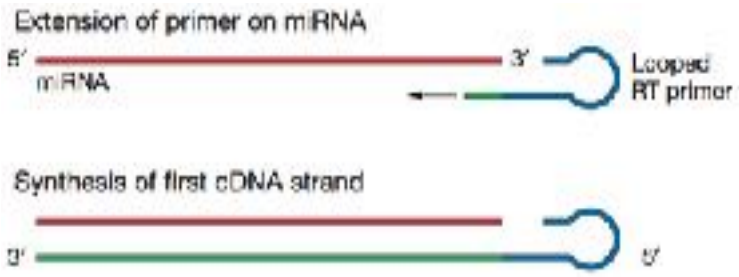


Figure 6: Illustration of cDNA synthesis reaction (adapted from TaqMan microRNA Reverse Transcription Kit protocol).

6. miRNA expression analysis

Measurement of miRNA expression was performed using specific TaqMan microRNA assays (Applied Biosystems). Target sequence of each miRNA as well as reference gene U6 snRNA are described in Table 2. Briefly, 4.5 µL of cDNA were transferred into each well of a 384-well plate and a mix of 0.5 µL of TaqMan assay and 5 µL of NZYSpeedy qPCR Probe Master Mix (NZYTech, Lisboa, Portugal) was added per well. Triplicates were performed for each sample. PCR reaction was performed in a LightCycler 480 Instrument (Roche Diagnostics, Manheim, Germany) within the following conditions: 3 min at 98ºC (enzyme activation); 45 cycles of 10 s at 95ºC followed by 25 s at 60ºC (denaturation, annealing, extension & acquisition) and 30 s at 37ºC (cooling; Figure 7).

Table 2: Target sequence of TaqMan microRNA expression assays.

Assay	Target Sequence
hsa-miR-182	5'-UUUGGCAAUGGUAGAACUCACACU-3'
hsa-miR-375	5'-UUUGUUCGUUCGGCUCGCGUGA-3'
U6 snRNA	5'-GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGA TACAGAGAAGATTAGCATGGCCCCCGCAAGGATGACACG CAAATTCGTGAAGCGTTCCATATTTT-3'

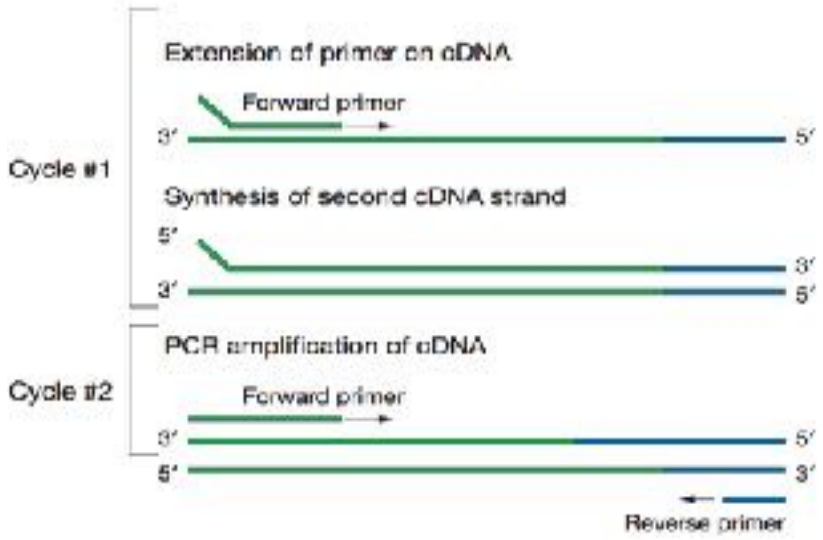


Figure 7: Illustration of qPCR reaction (adapted from TaqMan microRNA Reverse Transcription Kit protocol).

miRNA relative expression level was calculated by using comparative Ct method with U6 snRNA standing for as a reference gene. Relative expression was calculated under the following formula:

$$\textit{Relative miRNA expression} = 2^{-(Ct\ (miRNA) - Ct\ (U6\ snRNA))}$$

7. Statistical analysis

Statistical analysis of obtained data was performed using SPSS 24.0 software for Mac (IBM-SPSS Inc., Chicago, IL, USA). Graphics were built using GraphPad Prism 7.0 software for Mac (GraphPad Software Inc., La Jolla, CA, USA). A p-value inferior to 0.05 was considered statistically significant for 2-group comparisons, while Bonferroni correction ($p = 0.05 / n$) was applied for comparisons between more than two groups (n equals the number of groups).

Results

1. MicroRNA Expression from The Cancer Genome Atlas dataset

Our approach started by evaluating miRNA expression levels in malignant tissue and non-malignant prostatic tissue of patients that were retrieved from TCGA dataset. Comparison between tumor and normal samples as well as matched analysis between malignant and non-malignant tissue from the same patients were performed. Globally, miR-375 and miR-182 levels were significantly overexpressed in tumor compared to normal tissue. The same was found for paired PCa and normal samples (Figures 8-9).

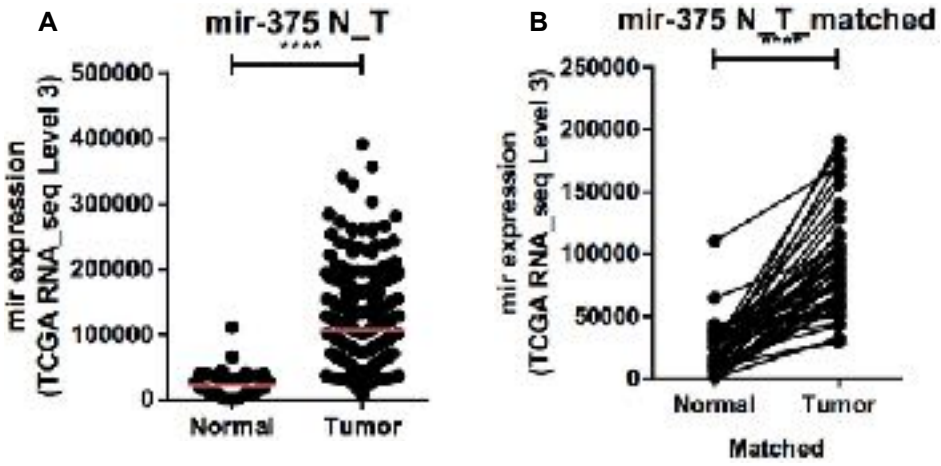


Figure 8: Expression of miR-375 in malignant and normal tissue from TCGA dataset. miR-375 was found to be overexpressed in tumor tissue both in overall(A) and matched (B) comparisons.

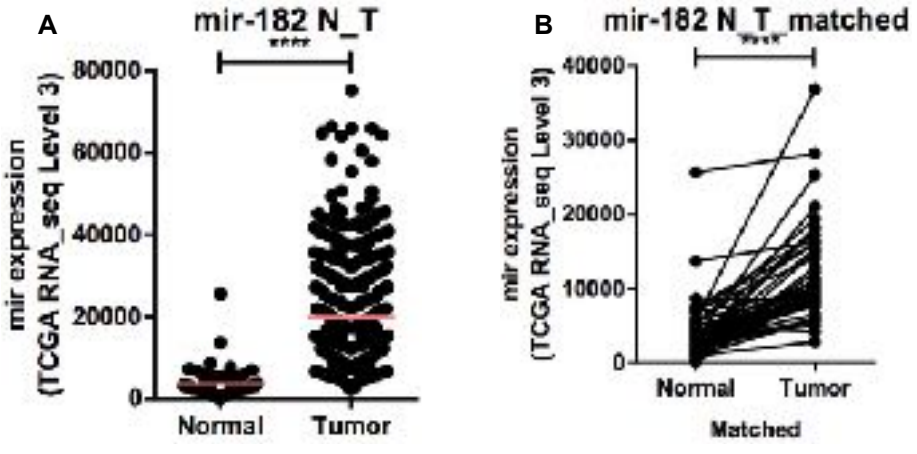


Figure 9: Expression of miR-182 in malignant and normal tissue from TCGA dataset. miR-182 was found to be overexpressed in tumor tissue both in overall(A) and matched (B) comparisons.

2. MicroRNA Expression in PCa Tissue Validation Cohort

To give further insight about the upregulation of the previous two miRNAs in PCa and their putative oncogenic role, a validation cohort of 100 patients diagnosed with PCa and treated with RP, and 15 controls (patients without prostate cancer, submitted to cystoprostatectomy due to bladder cancer) was built. Our approach consisted of analysing differences in expression between patients and individuals not harboring PCa as well as associations with clinicopathological variables. Clinical and pathological characteristics of patients included in this study are depicted in Table 3.

Table 3: Description of clinicopathological variables of patients included in this study.

Clinicopathological variables	Plasma Cohort (n = 210)	Matched Tissue Cohort (n = 100)	Healthy Tissue Cohort (n = 15)
Age (years), median (range)	**63** (46 - 76)	**63** (46 - 73)	**63** (45 - 80)
PSA at diagnosis (ng/mL), median (range)	**8.38** (0.68 - 42.66)	**8.8** (2.40 - 42)	**n.a.**
Follow-up time (days), median (range)	**2133** (117 - 6015)	**2776** (175 - 5704)	**n.a.**
Biochemical Recurrence (BCR)	**Yes:** 95 (45.2%) **No:** 115 (54.8%)	**Yes:** 48 (48.0%) **No**: 52 (52.0%)	**n.a.**
Metastasis	**Yes:** 18 (8.6%) **No:** 192 (91.4%)	**Yes**: 11 (11.0%) **No**: 89 (89.0%)	**n.a.**
Pathological Stage (pT)	**2**: 85 (40.5%) **3a**: 96 (45.7%) **3b**: 29 (13.8%)	**2:** 45 (45.0%) **3a:** 37 (37.0%) **3b:** 18 (18.0%)	**n.a.**
Grade Group	**1:** 49 (23.3%) **2:** 75 (35.7%) **3:** 55 (26.2%) **4:** 10 (4.8%) **5:** 21 (10.0%)	**1:** 28 (28.0%) **2:** 28 (28.0%) **3:** 27 (27.0%) **4:** 4 (4.0%) **5:** 13 (13.0%)	**n.a.**

n.a. = not applicable

2.1. Tumor and MNPT: Expression Differences

We started by comparing miRNA levels between tumor and morphologically normal prostatic tissue (MNPT). miRNA expression levels were compared through Mann-Whitney non-parametric test. Both miR-375 ($p = 0.0479$) and miR-182 ($p < 0.0001$) expression levels were significantly higher in tumor *vs* morphologically normal prostatic tissue (Figure 10).

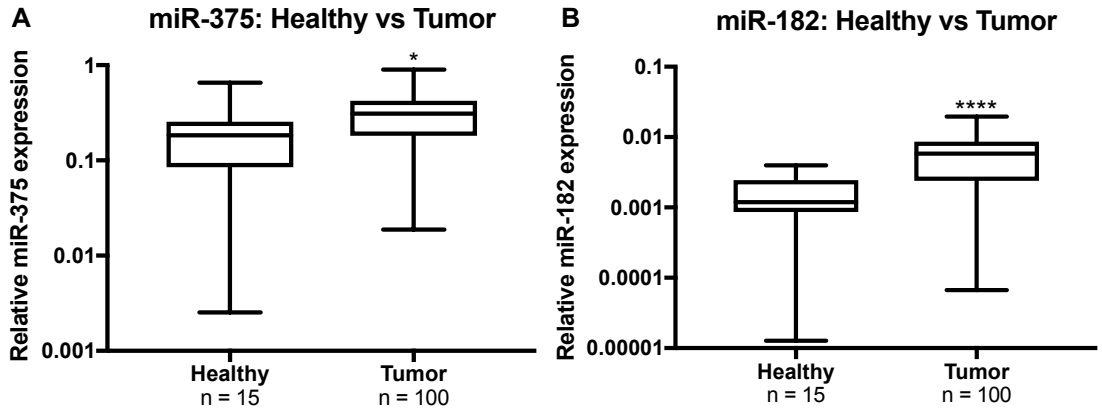


Figure 10: miR-375 (A) and miR-182 (B) expression levels in tumor and healthy tissue. Both miR-375 and miR-182 expression was significantly increased in tumor samples.

2.2. Correlation with Clinicopathological Variables

Next, we focused on analysing the relationship between tissue miRNA levels of our 100-patient cohort and the most relevant clinicopathological variables that represent features of the tumor specimen itself and patient-related variables at time of surgery. Gleason Grade Groups, Pathological Stage, preoperative PSA and age at time of surgery were selected.

The association between miRNA expression levels in PCa tissue and tumor aggressiveness, measured by the recently established Gleason Grade Groups was evaluated. The five different Grade Groups were merged into two categories: “low-grade” (GG 1-3) and “high-grade” (GG 4-5) and miRNA levels between both categories were compared through Mann-Whitney non-parametric test. Although miR-375 expression levels did not differ among patients with different grades ($p = 0.2735$), a trend for increased miR-182 levels in high-grade Groups ($p = 0.0845$) was found (see Annexes 1-2). Likewise, we searched for an association between miRNA expression levels and Pathological Stage (pT2, pT3a or pT3b) using Kruskal-Wallis (KW) one-way ANOVA test. However, no associations were found between miRs expression levels and pathological stage (miR- 375: KW: $p = 0.5523$; miR-182: KW: $p = 0.1656$; see Annex 3).

To assess correlation between tumorous miRNA levels and preoperative patients’ PSA levels, a linear regression model was built for each miRNA. Although miR-375 levels did not correlate with preoperative PSA ($p = 0.1451$), a significant inverse correlation was found between miR-182 expression and serum PSA levels ($\rho = 0.0151$; Figure 11).

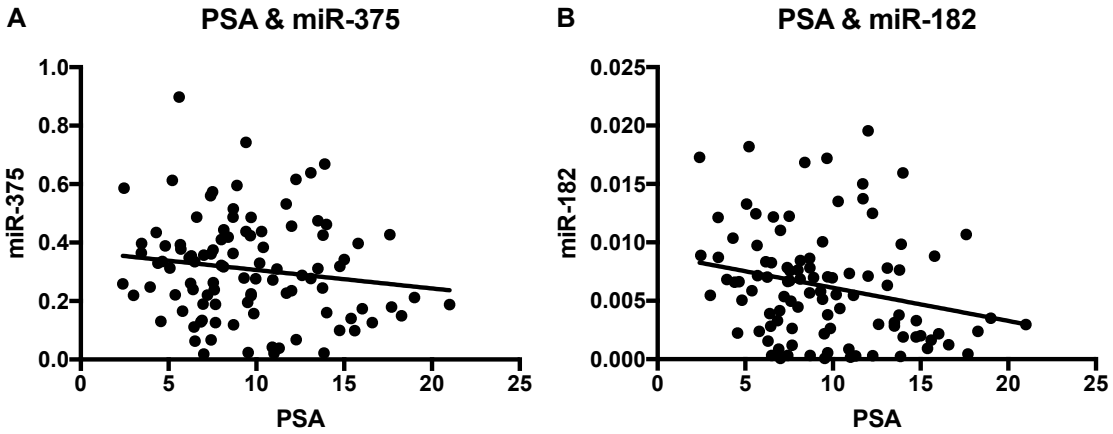


Figure 11: Linear regression of miR-375 (A) and miR-182 (B) levels and preoperative PSA value of each patient. A statistically significant negative correlation was observed between miR-182 and PSA.

A similar approach was used to analyse the relationship between miRNA levels and patients' age, but no correlation was found either for miR-182 ($p = 0.6092$) or miR-375 ($p = 0.9299$) (see Annex 4).

2.3. Survival Analysis

To determine the predictive potential of tumor-expressed miRNAs in respect to cancer-related late events such as BCR and metastasis, we started by measuring the effect of tumoral miRNA expression on BCR-free survival time through a Kaplan-Meier survival analysis with median miRNA expression level as cutoff. Log-rank test was used for comparison of survival curves. No statistically significant difference was found for miR-375 ($p = 0.4616$; Figure) or miR-182 ($p = 0.2658$ Figure) expression levels and Disease-Free Survival or Metastasis-Free Survival (miR-375; $p = 0.6553$ or miR-182; $p = 0.7630$; see Annexes 5-6).

Construction of a Cox proportional-hazards regression model was used to identify the variables that independently predict the occurrence of BCR. Grade Group ($p < 0.0001$) was the only statistically significant predictor BCR-free survival time. The remaining variables such as pT stage ($p = 0.3881$), age ($p = 0.9234$), preoperative PSA ($p = 0.2622$), miR-375 ($p = 0.9036$) and miR-182 ($p = 0.3636$) did not reach statistical significance. Accordingly, only High Grade group independently associated with shorter metastasis-free survival time ($p < 0.0001$), whereas no additional associations were found for any of the other studied variables [pT stage ($p = 0.2164$),

age (p = 0.7044), preoperative PSA (p = 0.9989), miR-375 (p = 0.2867) and miR-182 (p = 0.4772)].

3. Preoperative MicroRNA Expression in Plasma

After analysing miRNAs tumorous tissue expression, we evaluated miRNAs expression levels in preoperative and postoperative plasma of patients from our study cohort (n = 210). We started by analysing preoperative miRNA levels in plasma and searching for relevant associations with clinicopathological variables.

3.1. Correlation with Clinicopathological Variables

We sought to evaluate the association between preoperative miRNA levels and the recently established Grade Groups. We dichotomized samples into two categories: low (1-3) and high (3-5) Grade Groups. Data were analyzed using Mann-Whitney non-parametric test. Accordingly, no association was found between circulating miR-375 levels at time of RP and Grade Groups (p = 0.1616; Figure 12), whereas a significant increase of miR-182 levels was verified in High Grade Groups (p = 0.0310; Figure 13).

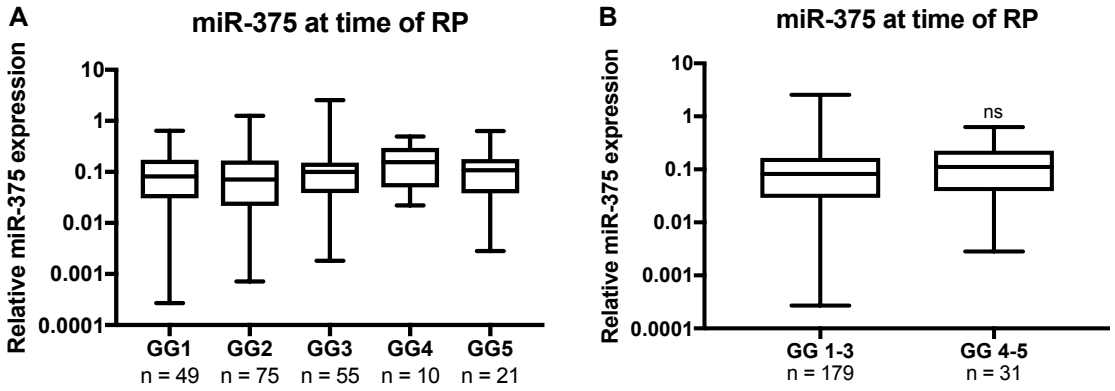


Figure 12: miR-375 expression levels stratified by Grade Groups (A) and dichotomized into low and high grade (B). No significant differences between high and low Grade Groups were observed.

The association between preoperative miRNA levels and Pathological Stage was assessed through Kruskal-Wallis one-way ANOVA test for comparison between three groups. Statistically significant differences for miR-375 (p = 0.0099) and for miR-182 (0.0192) were found between pT2, pT3a and pT3b. Pairwise comparisons performed with Mann-Whitney non-parametric test showed that preoperative miR-375

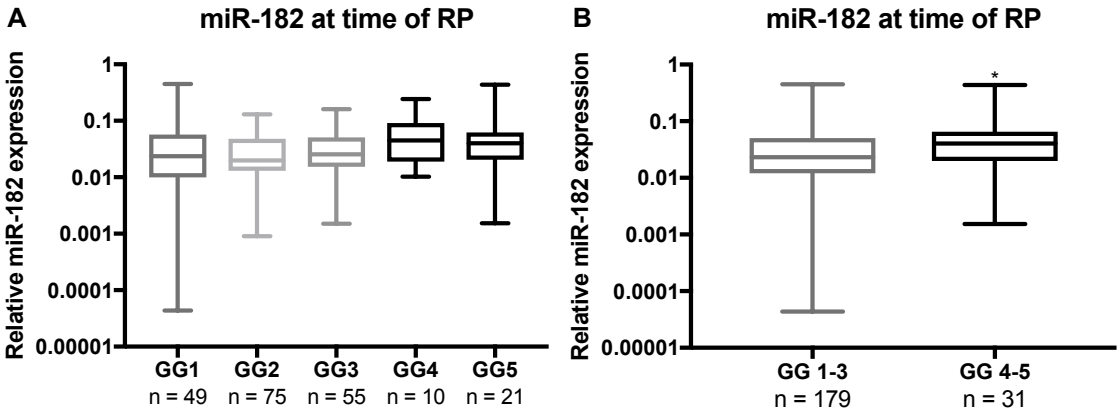


Figure 13: miR-182 expression levels stratified by Grade Groups (A) and dichotomized into low and high grade (B). A significant overexpression of plasma-circulating miR-182 was found in patients within higher Grade Groups.

levels in plasma were significantly increased among patients within pT3b comparing with pT3a (p = 0.0027) and pT2 (p = 0.0073). Moreover, significantly higher plasma-circulating miR-182 levels were displayed by pT 3b compared to pT 2 (p = 0.0098) patients (Figure 14).

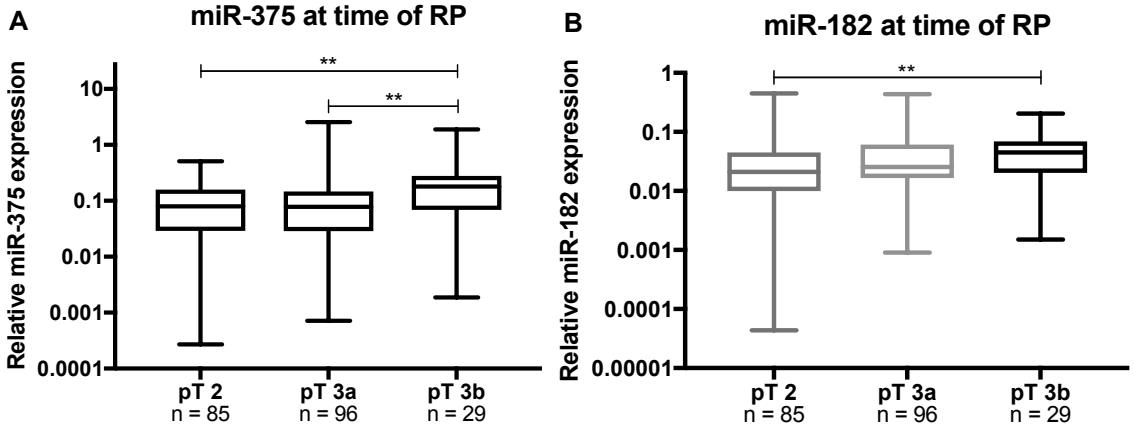


Figure 14: miR-375 (A) and miR-182 (B) expression levels stratified by pathological stage. A significant overexpression of both miR-375 and miR-182 was found in patients within higher stages.

A putative association between circulating miRNA levels and preoperative PSA levels in serum was similarly evaluated through the establishment of a linear regression model. However, the obtained slope was not statistically significant for miR-375 (p = 0.6993) or miR-182 (p = 0.0606; Annex 7). A similar approach was conducted for preoperative plasma-circulating miRNA levels and patients' age at the time of RP: a linear regression model was built, however the slopes were not statistically significant for miR-375 (p = 0.9353) or miR-182 (p = 0.4498; see Annex 8).

4. miRNA Plasma:tissue Ratio

After analysing the obtained levels of miR-375 and miR-182 in PCa tissue and plasma in a separate approach, we hypothesized that miRNA plasma:tissue ratio, *i.e.* tumor-normalized miRNA levels in plasma, might constitute a relevant indicator of disease extension and tumor aggressiveness as well as predictor of late progression events.

4.1. Correlation with Clinicopathological Variables

In order to evaluate the existence of an association between miRNA plasma:tissue ratio and tumor aggressiveness Grade Groups or dichotomized Grade Groups [Low grade (GG 1-3) and High grade (GG 4-5)] were compared for miRNA levels by Mann-Whitney non-parametric test. However, no statistically significant associations were observed for miR-375 ($p = 0.9855$) or miR-182 ($p = 0.5788$) ratios (see Annexes 9-10).

A similar approach was conducted for miRNA plasma:tissue ratio levels and pathological stage. Kruskal-Wallis one-way ANOVA test was used for a global comparison between three groups. Statistically significant differences were found for miR-375 ($p = 0.0022$) between pT2, pT3a and pT3b, but not for miR-182 ($p = 0.9985$). Pairwise comparisons with Mann-Whitney non-parametric test, demonstrated that miR-375 plasma:tissue ratio was significantly increased among patients with pT3b compared with pT3a ($p = 0.0028$), while pT3a patients presented significantly lower miR-375 plasma:tissue ratio levels compared with pT 2 ($p = 0.0110$). Conversely, no statistically significant associations were found between miR-182 plasma:tissue ratio levels and pathological stage (Figure 15).

Regarding miRNA plasma:tissue ratio no correlation with preoperative PSA and patients' age was found [PSA: miR-375 ($p = 0.1854$) and miR-182 (0.9537) (Figure); Age: miR-375 ($p = 0.1153$) or miR-182 ($p = 0.2333$)] (see Annexes 11-12).

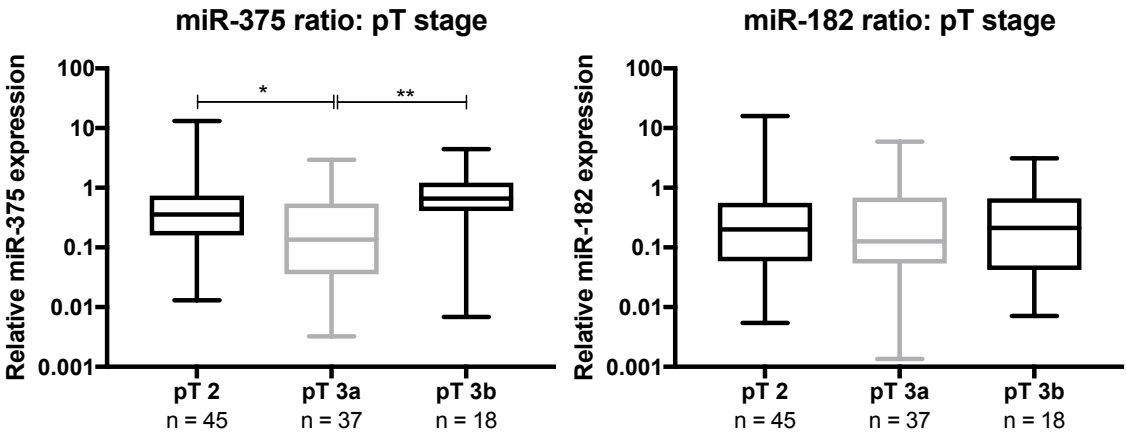


Figure 15: miR-375 (A) and miR-182 (B) plasma:tissue ratio levels stratified by pathological stage. Statistical significant differences were found for miR-375, but not for miR-182.

4.2. Survival Analysis

The prognostic value of miRNA plasma:tissue ratio regarding BCR was evaluated through the establishment of a Kaplan-Meier survival analysis. Patients were dichotomized into “High miR ratio” and “Low miR ratio” categories with median expression of each miRNA used as cut-off. Log-rank test was used for comparison of survival curves. High mR-375 ratio was significantly associated with shorter BCR-free survival time (p = 0.0344), whereas no significant association was observed regarding miR-182 (p = 0.1442; Figure 16). An identical approach was carried out for miRNA plasma:tissue ratio and metastasis-free survival time. However, no significant differences were observed for miR-375 (p = 0.4692) and miR-182 (p = 0.9268) ratios (see Annex 13).

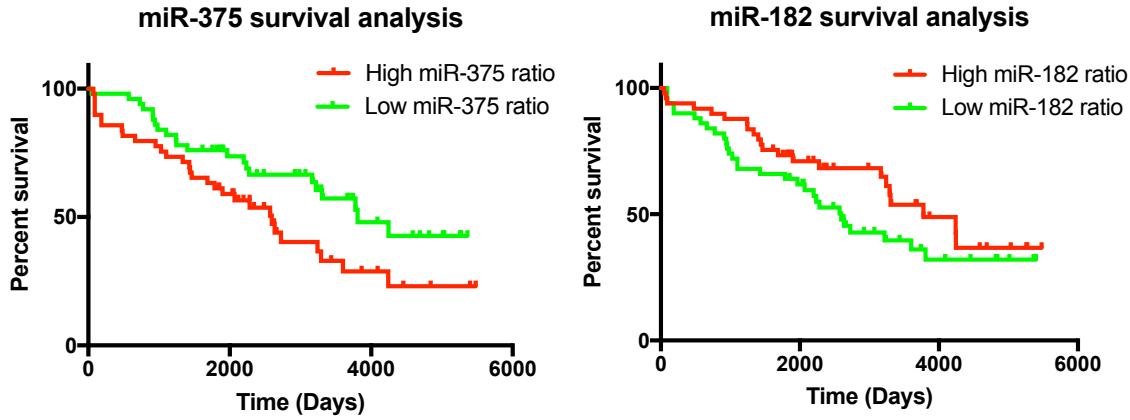


Figure 16: Kaplan-Meier BCR-free survival time of patients with high and low preoperative miR-375 (A) and miR-182 (B) plasma-tissue ratio. Patients with high miR-375 levels had significantly shorter BCR-free survival (p = 0.0344), while no significant association was registered for miR-182 (p = 0.1442).

With the goal of testing the relevance of miRNA ratio on survival time prediction compared to other clinicopathological variables, a Cox proportional-hazards regression model accounting for pT stage, Grade Group, age at diagnosis and PSA at diagnosis was performed. miR-375 ratio ($p = 0.0439$) and Grade Group ($p = 0.0002$) independently predicted the risk of developing BCR. The remaining variables such as pT stage ($p = 0.7088$), age ($p = 0.7461$), preoperative PSA ($p = 0.1322$) and miR-182 ($p = 0.452$) did not present statistical significance.

Regarding metastasis-free survival, Grade Group ($p < 0.0001$) significantly associated with this outcome, however pT stage ($p = 0.1581$), age ($p = 0.6699$), preoperative PSA ($p = 0.8196$), miR-375 ($p = 0.7034$) and miR-182 ($p = 0.5168$) did not significantly predict the development of metastasis.

5. Postoperative miRNA expression in Plasma

After studying circulating miRNA levels before RP, we focused on analysing miRNA levels after radical surgery and their putative value as markers of BCR and metastasis. We searched for differences in postoperative plasma-circulating miRNA levels according with patients' outcome after surgery. From 198 patients whose follow-up was obtained after outcome development, 115 did not display BCR ("no BCR"; control group), 70 presented BCR during follow-up ("BCR" group) and 13 developed metastatic disease ("metastasis" group). miR-375 and miR-182 expression levels among the three groups were compared through Kruskal-Wallis one-way ANOVA test and pairwise comparisons were performed using Mann-Whitney non-parametric test.

Kruskal-Wallis test ($p = 0.0055$) revealed significant differences for miR-375 across the three groups. Pairwise comparisons showed significantly higher miR-375 expression levels in plasma samples of patients that developed metastases ($p = 0.0034$) compared patients that did not progress. No additional significant differences were observed, although trends for increased miR-375 levels between 'BCR' and 'no-BCR' groups ($p = 0.0462$) and between 'metastasis' and 'BCR' patients ($p = 0.0570$) were observed. Contrarily, postoperative miR-182 levels did not significantly differ among the three groups (Figure 17).

A putative association between postoperative plasma-circulating miRNA levels and concomitant PSA levels was evaluated through construction of a linear regression

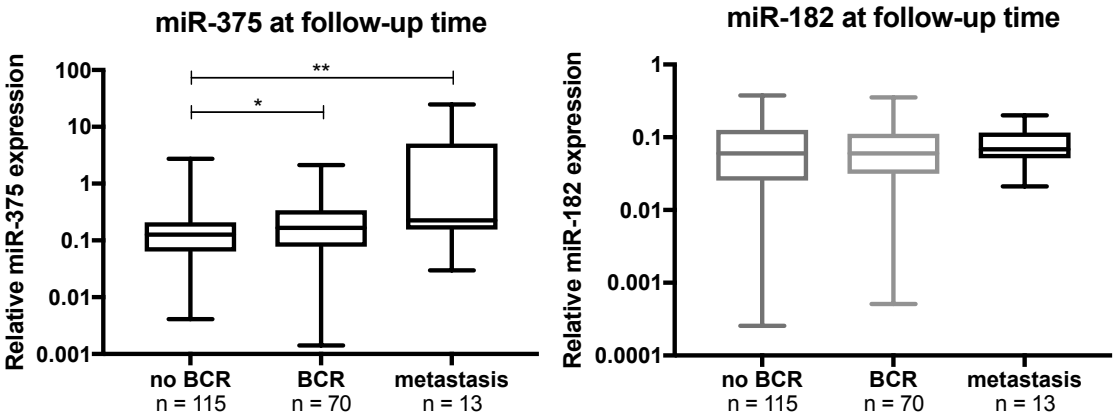


Figure 17: Levels of postoperative plasma-circulating miR-375 (A) and miR-182 (B) according to the developed outcome after surgery. Significant differences were registered for miR-375, but not miR-182.

model. A statistically significant positive correlation with PSA was obtained for miR-375 ($p < 0.0001$), but not for miR-182 ($p = 0.7210$; Figure 18).

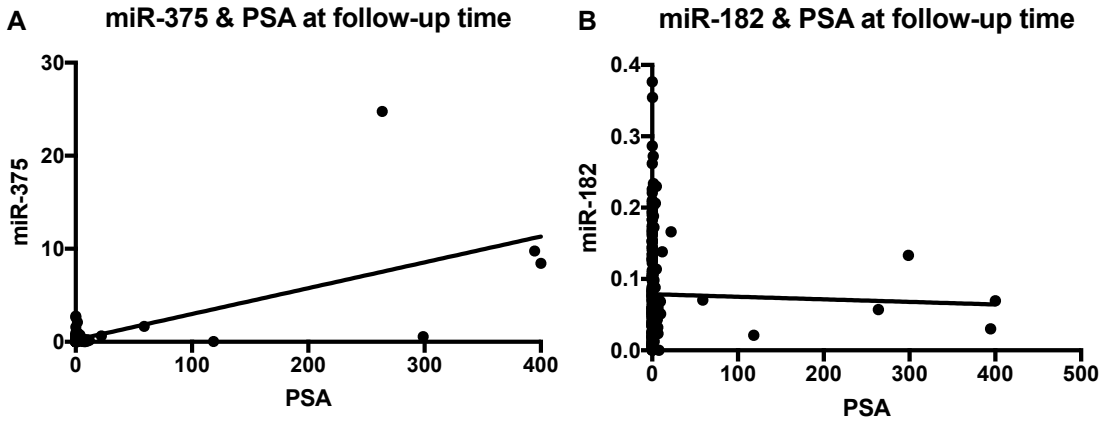


Figure 18: Linear regression of miR-375 (A) and miR-182 (B) levels and postoperative PSA value of each patient. A statistically significant positive correlation was observed between miR-375 and PSA.

In a similar approach, a correlation between postoperative plasma-circulating miRNA levels and the age of each patient at the same time point was evaluated through linear regression and Pearson coefficient determination. No statistically significant correlations were observed for miR-375 ($p = 0.8564$) or miR-182 ($p = 0.7078$; see Annex 14).

Discussion

Since their first report in the context of malignant disease in 2002, miRNAs have shown enormous potential as cancer biomarkers. Particularly, the stable circulation of miRNAs in body fluids such as plasma has been consistently described since 2008 and greatly broadens the usefulness of miRNAs as biomarkers in a time when great efforts are being made for the identification of new non-invasive methods of diagnosis, prognosis and personalized follow-up.

In a previous study from our research group, which constitutes the basis for this dissertation, a panel of 17 miRNAs were found to be overexpressed in PCa tissue compared to matched normal prostate tissue (MNPT) by microarray analysis. miR-182 and miR-375 were then selected for further studies and miR-182 and miR-375 overexpression in PCa (n = 80) *vs* MNPT (n = 15) was validated. We firstly decided to obtain further evidence of miRNA deregulation in malignant tissue by assessing miR expression both in the publicly available TCGA genomic dataset and in a larger patient cohort. Indeed, we confirmed that miR-375 and miR-182 were overexpressed in PCa cases from TCGA dataset compared to normal controls both globally as well as in matched tissues comparison. Similarly, miR-375 and miR-182 were overexpressed in PCa tissues from our 100-patient cohort compared to MNPT controls, thereby supporting their previously described oncogenic role in prostate carcinogenesis [141].

Because the main goal of this study was to evaluate variations in patient miRNA expressing profile during follow-up after radical prostatectomy, it seemed pertinent to assess if selected miRNAs were not only overexpressed in patients *vs* healthy controls but also similarly upregulated in tumors with different clinicopathological features. However, no statistically significant differences were found for miR-375 among patients within different Grade Groups or Pathological Stage and no correlations with age at time of surgery and preoperative PSA were established, either, suggesting that miR-375 levels do not reflect the extension and the histological aggressiveness of the tumor. Survival analysis did not disclose any significant association between miR-375 expression and BCR- and metastasis-free survival time. Likewise, no statistically significant associations were found between miR-182 levels and pathological stage, preoperative PSA and age at time of diagnosis, disease- and metastasis-free survival excepting a trend for higher miR-182 expression in patients within higher Grade Group. Therefore, both miR-375 and miR-182 arise as putative oncomiRs in the context of PCa since their expression is

increased in tumor cells compared to non-neoplastic prostatic cells, although miR-375 and miR-182 levels in PCa do not associate with standard parameters predictive of clinical aggressiveness.

Subsequently, we measured miR-375 and miR-182 circulating levels (plasma) in a 210-patient cohort in two different time points: immediately before RP and during patient follow-up. We started by analysing preoperative miRNA levels and their association with clinicopathological features. Interestingly, circulating miR-375 levels were significantly higher in patients staged pT3b compared to those staged pT3a and pT2. Circulating miR-182 were also found upregulated in patients staged pT 3b *vs.* pT2 and miR-182 overexpression in patients with higher grade group was similarly found. No additional statistically significant results were observed. Recalling that pTb represents extraprostatic extension through invasion of seminal vesicles, conveying a high risk of PCa dissemination, miR-375 and miR-182 upregulation in plasma might reflect increased tumor burden and, eventually, the occurrence of imagiologically undetected metastatic disease. Therefore, and contrarily to their expression patterns in tissue, miR-375 and miR-182 in plasma may represent early markers of PCa progression.

Due to the fact that both miRNAs (miR-375 and miR-182) present distinct associations with clinicopathological parameters depending whether expression is evaluated in PCa tissue or plasma, we hypothesized that miRNA plasma:tissue ratio, an indicator of tumor-normalized miRNA levels in circulation, might constitute an indicator of disease extension and tumor aggressiveness as well as a predictor of late progression events. Interestingly, we found that miR-375 ratio is significantly increased in patients within stage pT3b *vs* stage pT3a. Moreover, survival analysis revealed that patients with high miR-375 ratio display significantly shorter BCR-free survival time. Building of a Cox proportional-hazards regression model accounting for the remaining parameters confirmed that miR-375 ratio independently predicts for risk of BCR. Contrarily, we did not find any statistically significant associations between miR-182 ratio and clinicopathological parameters. We hypothesized that measurement of miRNA plasma:tissue ratio could add prognostic value to miR-375, being a potentially more relevant prognostic indicator than miRNA levels in plasma or tissue *per se*. However, miR-182 ratio does not match the associations found for plasma-circulating miR-182, which could suggest that miR-182 might not have prognostic value on its own and its levels in plasma mostly reflect tumor extent before surgery.

To clarify our hypotheses, we sought to analyse miRNA expression in plasma during patient follow-up, after RP, and to determine whether their expression predicts for late progression events such as BCR or metastasis. Among our 210-patient cohort, 115 patients did not developed recurrence, 77 patients developed BCR without metastasis and 18 patients developed metastasis. For this analysis, patients whose follow-up plasma was obtained before the development of each outcome (BCR or metastasis) were excluded, since miRNA levels could not chronologically reflect the presence of the outcome. Thus, seven patients from BCR subgroup and five patients from metastasis subgroup were excluded and miRNA levels from 198 patients were analysed. Interestingly, miR-375 levels were higher in patients that experienced BCR or metastization, although statistical significance was only reached for the latter, the former only disclosed a trend. Furthermore, a positive correlation was found between plasma-circulating miR-375 and postoperative PSA levels, which are routinely used to monitor PCa patients. Thus, it will be interesting to determine whether miR-375 might complement or eventually replace serum PSA as a follow-up biomarker.

Indeed, the observed global trend of higher levels among patients with worse outcome, strongly suggests that miR-375 may increase during follow-up time in parallel with PCa progression and may, thus, constitute a novel biomarker. Positive correlation with serum PSA, the only currently used biomarker for PCa progression after radical treatment, further supports the use of miR-375 as a disease marker. Moreover, this finding is in agreement with the predictive potential of miR-375 ratio, suggesting that miR-375 plasma:tissue ratio reflects tumor extension and predicts BCR-free survival, whereas postoperative plasma-circulating miR-375 might be a marker of disease progression. Contrarily, the results disclosed for miR-182 suggest that miR-182's role in prostate carcinogenesis should be mostly during early stages of PCa development rather than disease progression after treatment.

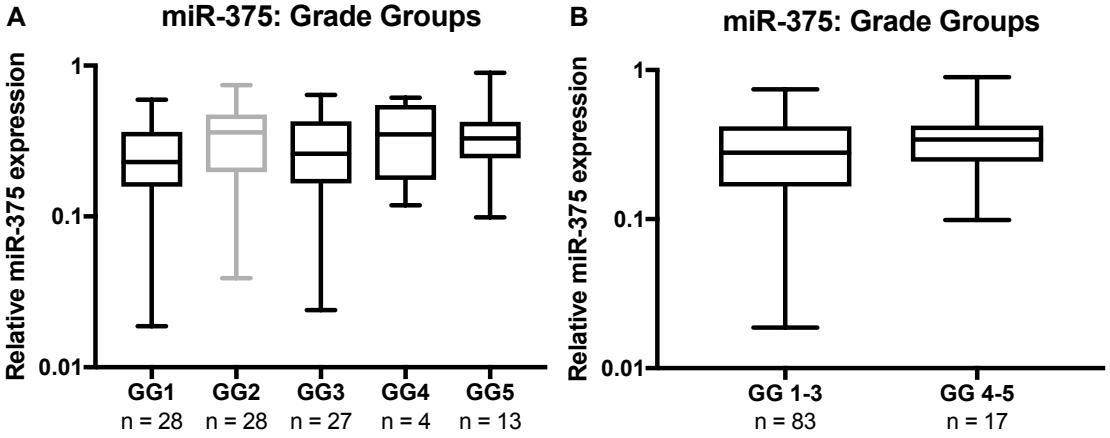
Conclusions & Future Perspectives

Comparing the obtained results with the previously published reports, we suggest that miR-182 plays an oncogenic role in the earliest steps of PCa progression due to its overexpression in malignant tissue vs MNPT. Plasma-circulating levels of miR-182 reflect early dissemination stages of PCa, however the lack of prognostic value or miR-182 plasma:tissue ratio before RP as well as the absence of a significant association between postoperative miR-182 levels in plasma and disease progression after radical surgery suggests that miR-182 may act as an oncomiR in early PCa progression stages rather than a marker of disease progression after treatment.

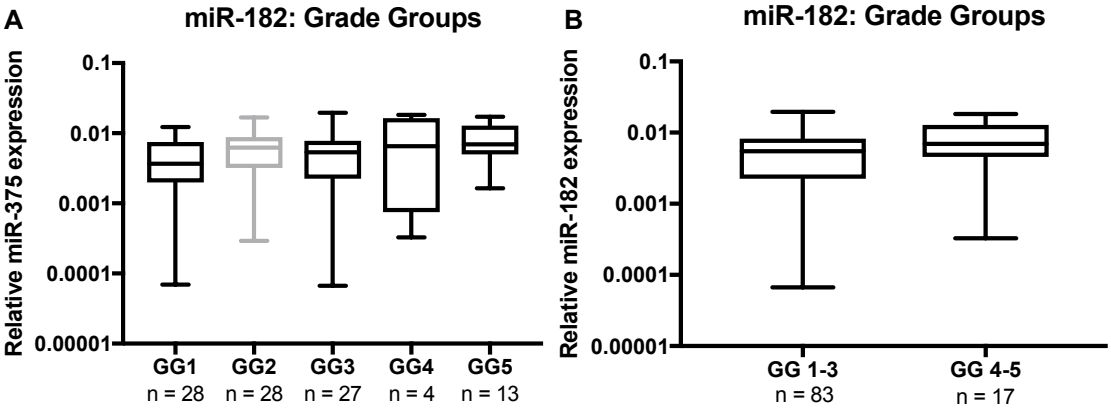
Contrarily, our results suggest that miR-375 might be implicated in the earliest stages of PCa but also constitute an independent predictor of BCR and a marker of postoperative disease progression, thus, potentially able to complement and/or replace serum PSA as a follow-up biomarker. Moreover, measurement of miR-375 plasma:tissue ratio before surgery, rather than plasma-circulating levels *per se*, may assist in prognostic assessment to currently used tools.

For the purposes of future research we suggest that systematic collection of peripheral blood during patient follow-up and the respective measurement of miR-375 levels could be helpful to obtain further evidence of miR-375 performance as a follow-up biomarker, since it would enable the construction of a miR-375 expression profile throughout follow-up time that might predict the occurrence of late events such as BCR and metastasis and therefore allow an early intervention that could increase patient survival and thus improve their quality of life.

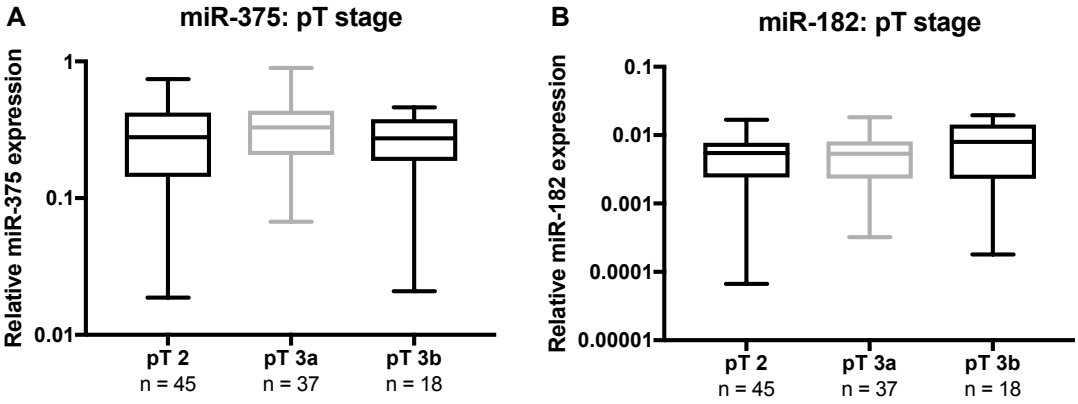
Annexes



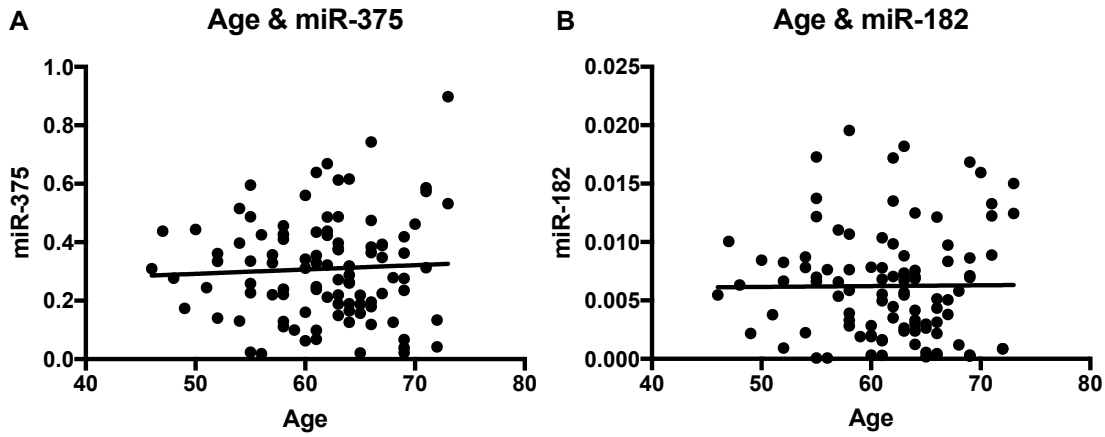
Annex 1: miR-375 expression levels stratified by Grade Groups (A) and dichotomized into low and high grade (B). No significant differences between high and low Grade Groups were observed.



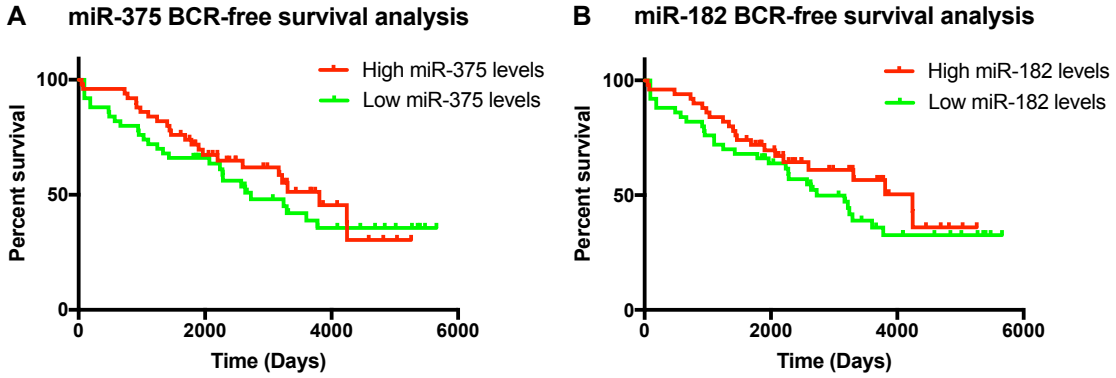
Annex 2: miR-182 expression levels stratified by Grade Groups (A) and dichotomized into low and high grade (B). A tendency of overexpression was found in patients within higher Grade Groups.



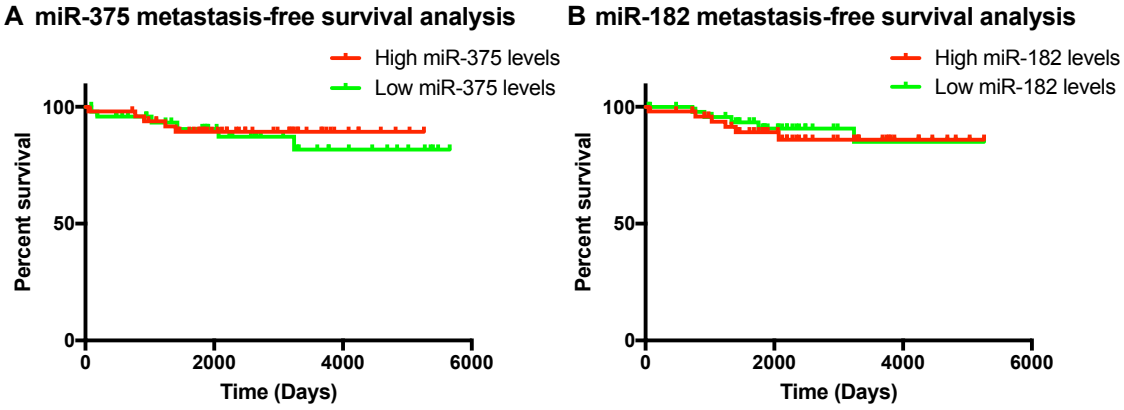
Annex 3: miR-375 (A) and miR-182 (B) expression levels stratified by pathological stage. No statistically significant differences were found.



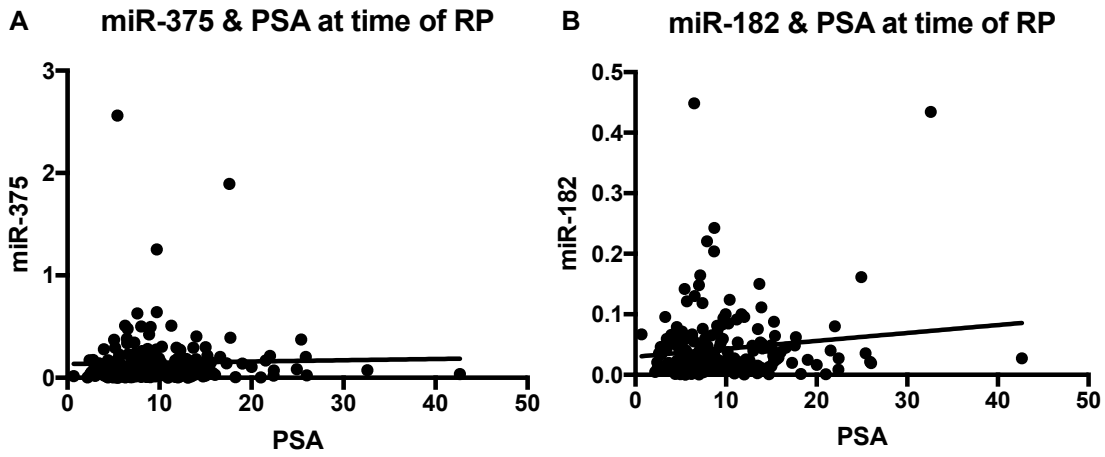
Annex 4: Linear regression of intra-tumoral miR-375 (A) and miR-182 (B) levels and age of each patient at time of RP. No statistically significant correlations were observed.



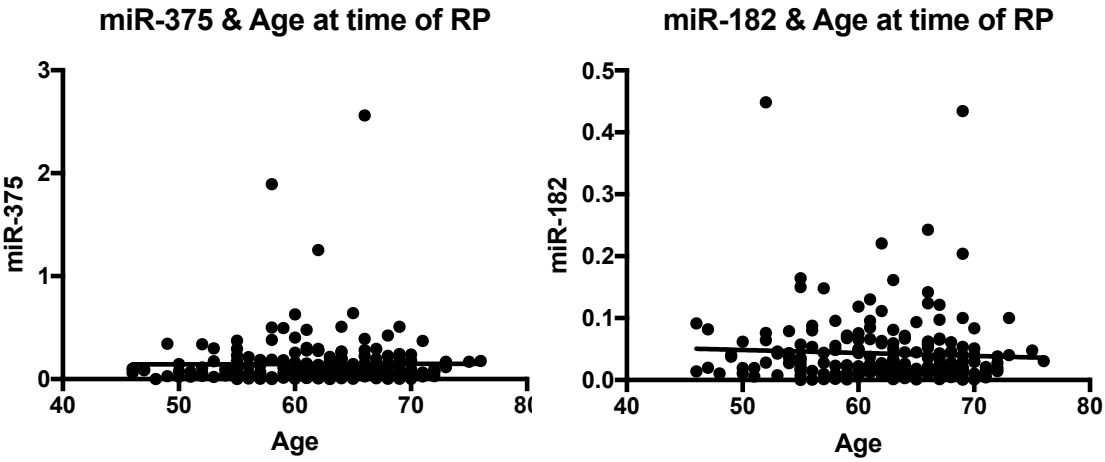
Annex 5: Kaplan-Meier BCR-free survival of patients with high and low intratumoral miR-375 (A) and miR-182 (B) levels. No statistically significant differences between survival curves were obtained.



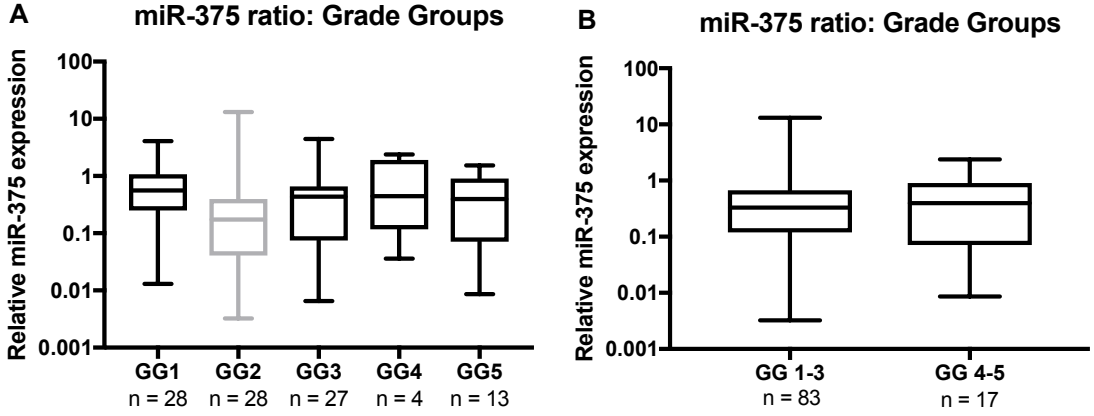
Annex 6: Kaplan-Meier metastasis-free survival of patients with high and low intra-tumoral miR-375 and miR-182 levels. No statistically significant differences between survival curves were obtained.



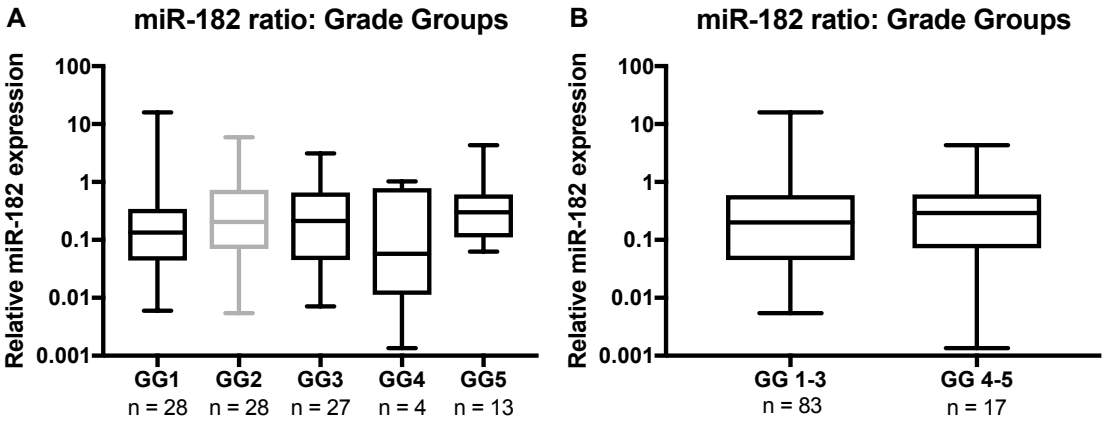
Annex 7: Linear regression of miR-375 (A) and miR-182 (B) levels and preoperative PSA value of each patient. No statistically significant correlations were observed.



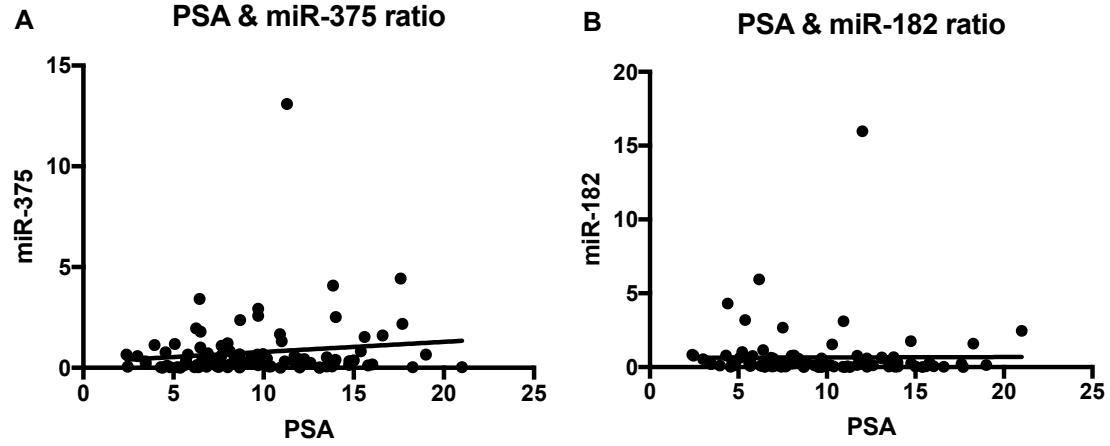
Annex 8: Linear regression of miR-375 (A) and miR-182 (B) levels and age of each patient at time of RP. No statistically significant correlations were observed.



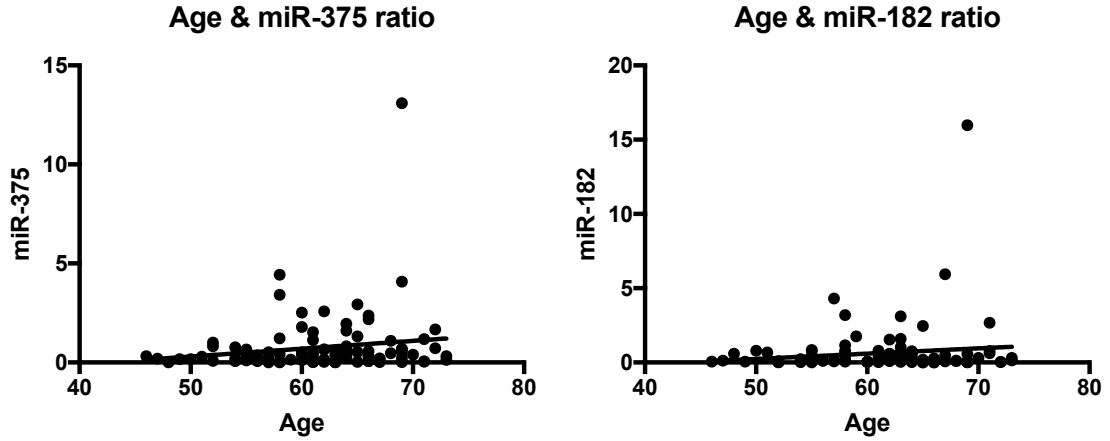
Annex 9: miR-375 plasma:tissue ratio levels stratified by Grade Groups (A) and dichotomized into low and high grade (B). No significant differences between high and low Grade Groups were observed.



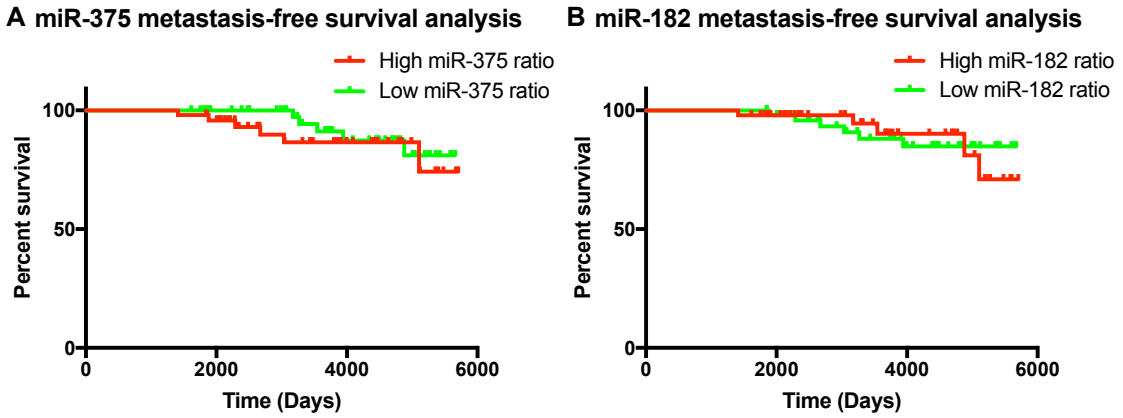
Annex 10: miR-182 plasma:tissue ratio levels stratified by Grade Groups (A) and dichotomized into low and high grade (B). No significant differences between high and low Grade Groups were observed.



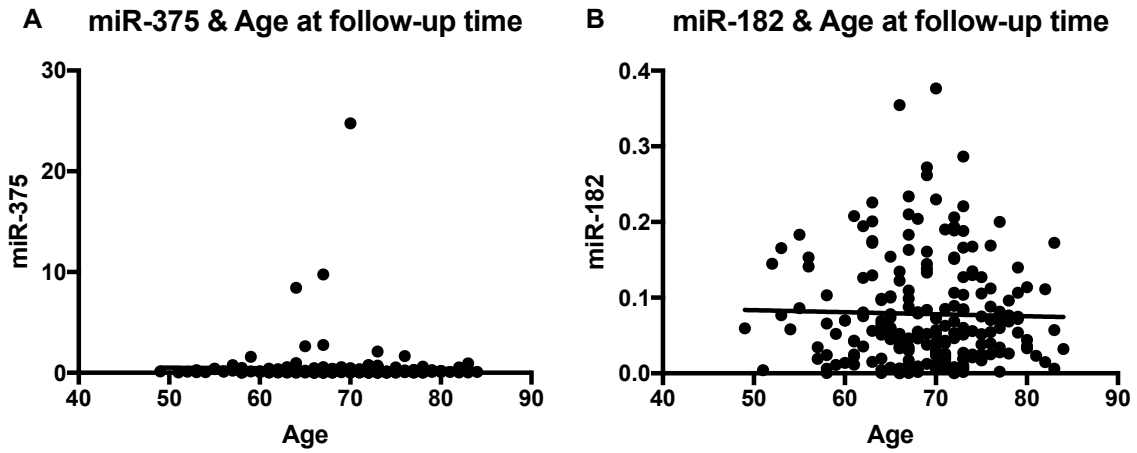
Annex 11: Linear regression of miR-375 (A) and miR-182 (B) levels and preoperative PSA value of each patient. No statistically significant differences were obtained.



Annex 12: Linear regression of miR-375 (A) and miR-182 (B) levels and age of each patient at time of RP. No statistically significant differences were obtained.



Annex 13: Kaplan-Meier BCR-free survival time of patients with high and low preoperative miR-375 (A) and miR-182 (B) plasma-tissue ratio. No statistically significant associations were found.



Annex 14: Linear regression of miR-375 (A) and miR-182 (B) levels and age of each patient at follow-up time. No statistically significant correlations were observed.

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